Blood biomarkers and novel imaging techniques in acquired heart disease

Sharda Suzan Anroedh

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Thesis

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by
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born in Paramaribo (Suriname)

Erasmus University Rotterdam

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ॐ असतो मा सद्गमय ।
तमसो मा ज्योतिर्गमय ।
मृत्योर्मा अमृतं गमय ।
ॐ शान्तिः शान्तिः शान्तिः ॥

Om Asato Maa Sad-Gamaya |
Tamaso Maa Jyotir-Gamaya |
Mrtyor-Maa Amrtam Gamaya |
Om Shaantih Shaantih Shaantih ||

Lead us from the unreal to the real
Lead us from darkness to light
Lead us from death to immortality
Aum peace, peace, peace!

Especially for my parents and Parisha

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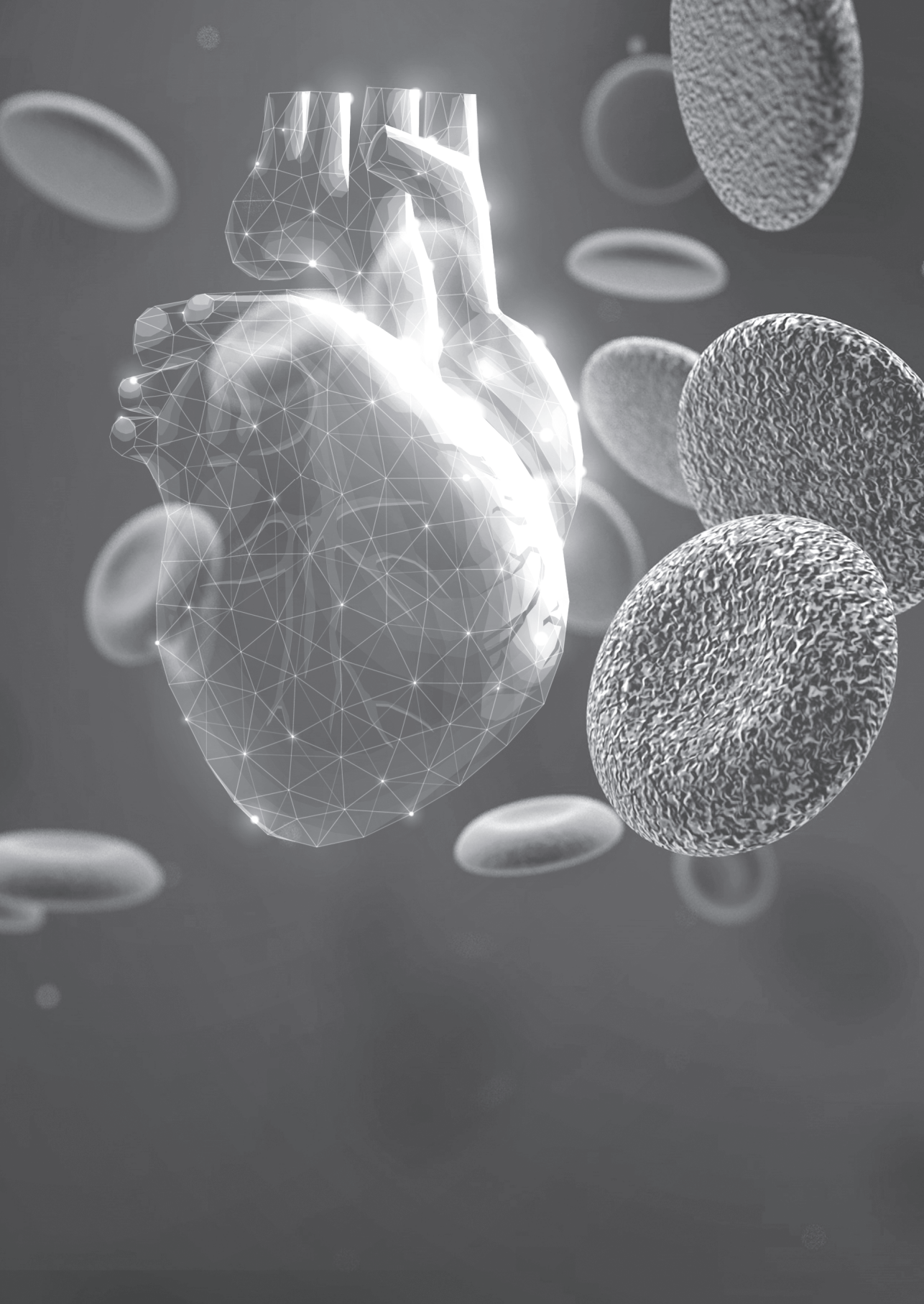
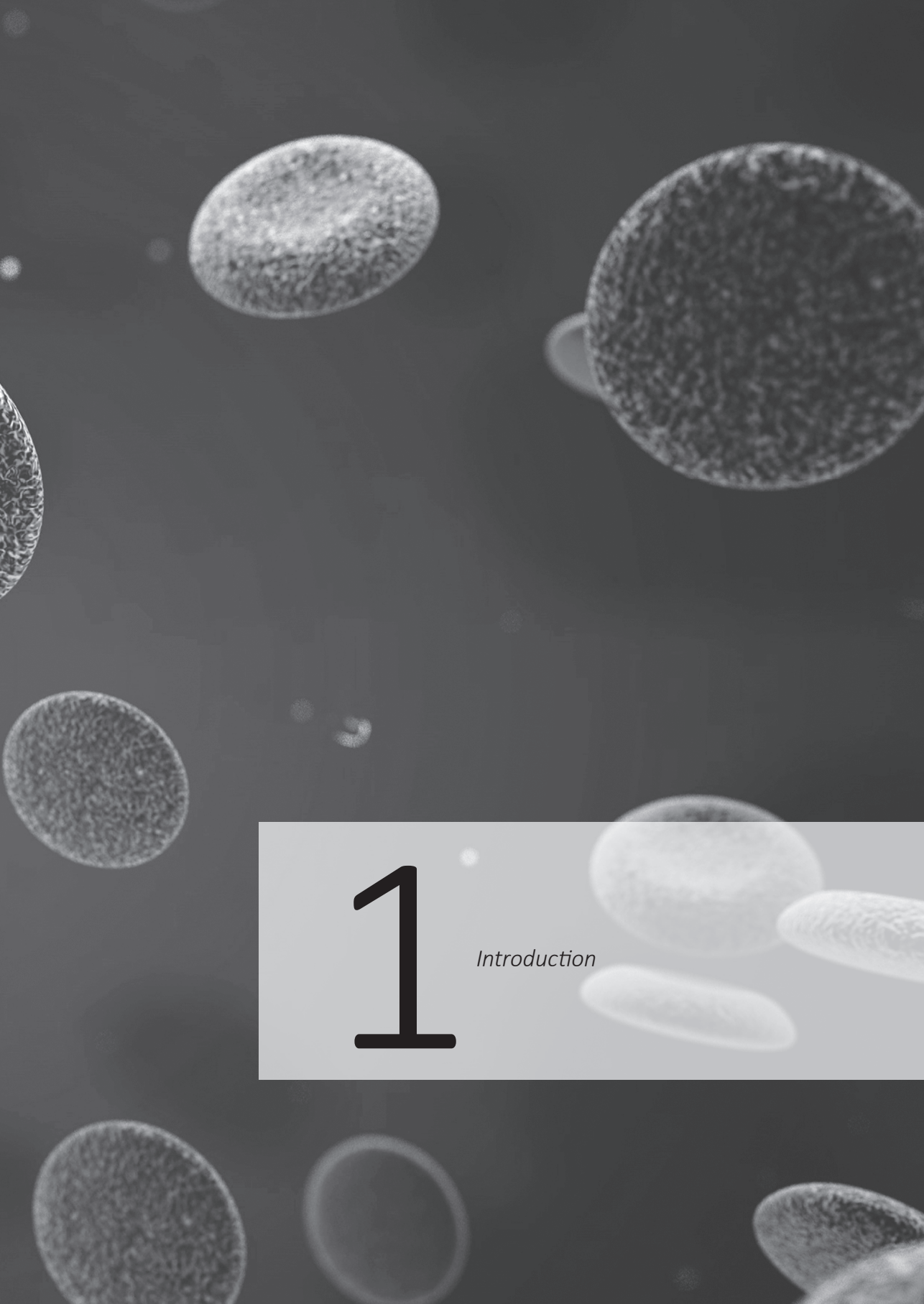




1 *Introduction*

INTRODUCTION

Burden of cardiovascular disease

Although their treatment has drastically improved in the past decades, cardiovascular diseases (CVD), or diseases of the circulatory system (ICD-10 code I00-I99), still remain the most common cause of death worldwide, with more than 4 million people dying in Europe every year (1). This may in part be attributed to the ageing of the general population, for which reason mortality rates due to CVD are expected to rise even further in the future (2). With 37,795 (24.6%) deaths, CVD / diseases of the circulatory system were the second cause of mortality in The Netherlands in 2018 (Figure 1). Taking a closer look, within the class of CVD, coronary artery disease (CAD; ICD-10 I20-I25) and heart failure (HF; ICD-10 I50-I51) caused 8,268 and 7,564 deaths, respectively (3), whereas CAD and HF were responsible for 67,607 and 30,405 hospital admissions (4). These numbers underscore the burden of CVD on Dutch society, in particular CAD/HF, and call for continuing action.

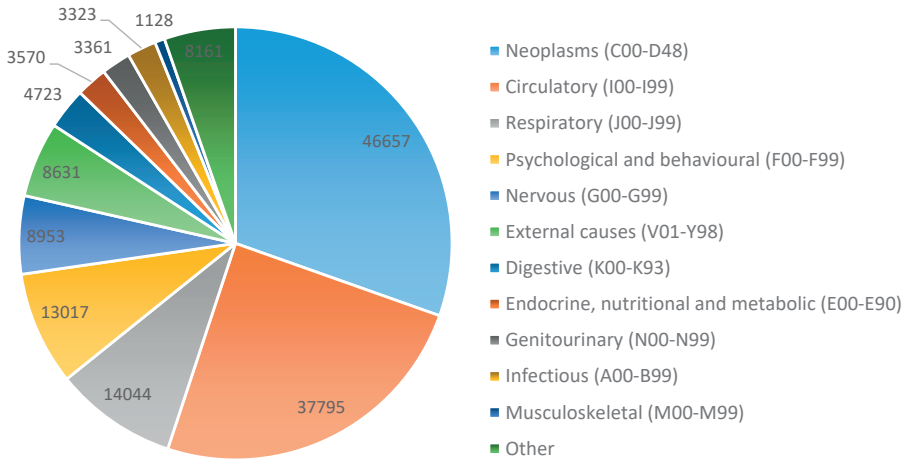


Figure 1 - Causes of mortality in The Netherlands in 2018 according to the ICD-10 classification (source: https://opendata.cbs.nl)

Understanding the disease course in individuals with acquired heart disease

In order to reduce the burden of CAD/HF on patients and society, a combination of public prevention campaigns with a personalized approach is required. In order to fully utilize the opportunities of a personalized medicine model, obviously, our understanding of the disease should be improved, in particular the prediction of

an adverse disease course in individual patients. This thesis aimed to investigate if and to what extent serum biomarkers and intracoronary imaging may contribute to these goals in patients with established CAD (Part I) and HF (Part II).

Part I - Coronary artery disease

In the first part of this thesis we focus on patients with established CAD, and study: the relation between the anatomic Syntax score (SXscore), based on coronary angiography (CAG), and coronary plaque characteristics as assessed by radiofrequency intravascular ultrasound (RF-IVUS) and near-infrared spectroscopy (NIRS) imaging; the relation between serum markers of inflammation, renal function and lipid metabolism, and the NIRS-derived coronary lipid core burden index (LCBI); changes in serum CRP levels in relation to changes in blood lipids and coronary plaque characteristics in patients receiving high-intensity statin treatment; the association between biomarkers of inflammation, renal function and lipid metabolism with the occurrence of major adverse cardiac events (MACE) during long-term follow-up.

The outline of this section is as follows:

In **Chapter 2** we study 680 CAD patients, and examine the association between the atherosclerotic burden derived from all three coronary arteries, as assessed by the CAG-based SXscore, and the atherosclerotic burden as assessed by RF-IVUS and NIRS in a single, non-culprit segment. The SXscore is a well-established anatomical scoring tool that grades the complexity of the luminal coronary obstruction and is also used for short- and long-term prediction of MACE in patients undergoing revascularization (5, 6). This score takes into account the number of significant lesions and their location, as well as the complexity of each lesion independently. We investigate whether information derived with this established tool is correlated with information on the extent and phenotype of coronary atherosclerosis as provided by the intracoronary imaging modalities RF-IVUS and NIRS. RF-IVUS is capable of identifying thin-cap fibroatheroma (TCFA) lesions, which are predictive for the occurrence of MACE, particular death and acute coronary syndrome (ACS) (7). NIRS is capable of identifying plaques in the coronary wall with a lipid rich core, which are vulnerable for rupture (8, 9).

In CAD patients, NIRS and FR-IVUS derived plaque characteristics contain prognostic information. Circulating biomarkers are also useful to identify CAD patients who are prone to an adverse disease course. Moreover, serum biomarkers may be

capable to detect vulnerable coronary plaques in an early stage and in a non-invasive manner. Investigating blood biomarkers in relation to intravascular imaging findings could help bridge the gap between known biological pathways and clinical characteristics of atherosclerosis, and could provide further insights into prognostication. Thus, in the **chapters 3, 4, and 5** we study circulating biomarkers of inflammation and renal function, and lipid metabolism, in 581 CAD patients who participated in the ATHEROREMO study. We investigate the relationship between these biomarkers and NIRS-derived LCBI, as well as their association with the occurrence of MACE during longer-term follow-up.

In **chapter 6** we focus further on inflammation, which is known to play a major role in the initiation, progression, and instability of atherosclerotic plaques (10, 11). Among all inflammatory biomarkers, C-reactive protein (CRP) in particular, has been extensively investigated and proven as a prognostic biomarker of CVD (12). Here, we examine the associations between serially measured serum CRP levels with changes in cholesterol levels and changes in intracoronary plaque characteristics as assessed by RF-IVUS and NIRS in a series of 164 patients who received intensive statin therapy for 1 year.

Finally, in **chapter 7**, we move to the clinical practice of treating CAD patients. Here our focus is on patients presenting with chest pain suggestive of myocardial infarction (MI). In pre-hospital settings handled by paramedics, appropriate triage of MI patients remains challenging when automated electrocardiogram (ECG) interpretation is inconclusive. We aimed to identify those patients in order to get them on the right track to primary percutaneous coronary intervention (PCI). For that purpose, In the Rotterdam-Rijnmond region, automated ECG devices on all ambulances were supplemented with a modem, enabling transmission of ECGs for online expert interpretation. The diagnostic protocol for acute chest pain was modified accordingly. We monitored the performance of this system during 1 year, and report on a total of 1,076 patients.

Part II - Heart failure

For HF patients several multivariable prognostic risk scores have been developed. These scores mostly rely on clinical characteristics, and leave room for improvement. In the past years a large body of research has emerged showing that many circulating biomarkers are involved in heart failure (13, 14). However, previous studies on this topic ‘classically’ related single biomarker measurements at study baseline with adverse events occurring over the years thereafter. Thus, disease dynamics

might easily have been missed. We hypothesized that temporal biomarker patterns contain additional prognostic information that may help improve individualized risk assessment.

In the second part of this thesis we focus on patients with established HF. We investigate temporal patterns of a broad range of serum biomarkers, including markers of inflammation, myocardial ischemia, myocardial stress, microRNAs, and glomerular and tubular renal markers; and relate these temporal patterns with the clinical (adverse) disease course.

The outline of this section is as follows:
In the **chapters 8 and 9** we study a series of 263 ambulant HF patients who had blood sampling at enrolment and subsequently every 3 months. We examine the associations between repeatedly measured N-terminal pro-B-type natriuretic (NT-proBNP), high-sensitivity troponin T (Hs-TnT), C-reactive protein (CRP), which are established prognostic biomarkers in HF patients (13), and New York Heart Association (NYHA) class, which classifies symptom severity of HF. We also examine the association between the temporal evolution of these biomarkers and occurrence of adverse events, including cardiovascular (CV) mortality and HF hospitalisation.

Several studies have suggested that circulating microRNAs (miRs) are associated with HF, but these studies were small, and limited to single miR measurements. In **chapter 10** we examine 7 miRs that were previously linked to heart failure, and test whether their temporal expression level predicts the incidence of CV mortality or HF hospitalisation in the above-mentioned prospective cohort of ambulant HF patients.

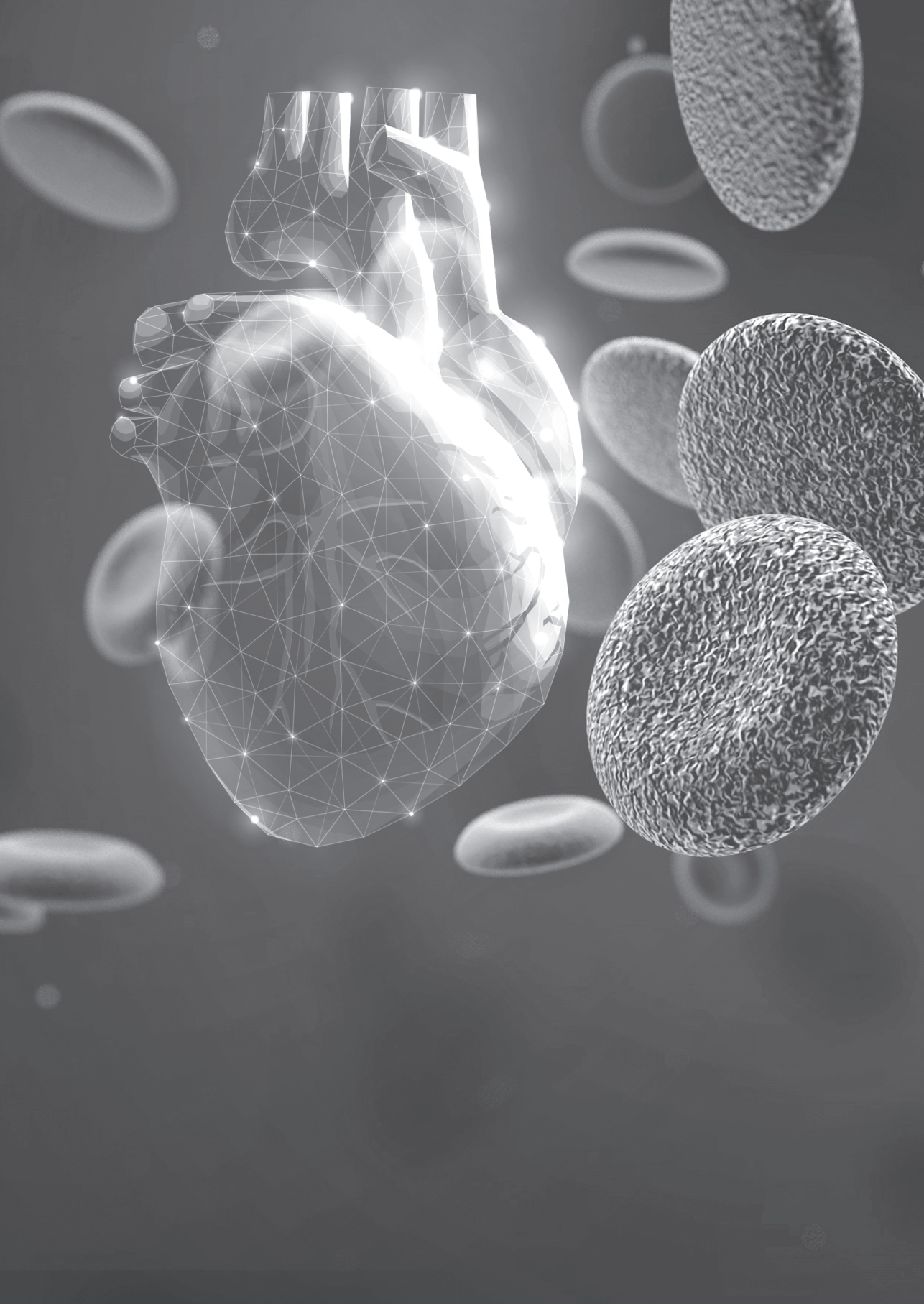
Heart failure patients often have impaired renal function (13, 15), whereas renal (dys)function itself is an important determinant of adverse clinical outcome. Single assessments of renal function fail to reflect clinically silent progression of HF. Therefore, in **chapter 11**, we again studied the 263 ambulant HF patients, and now evaluated the temporal evolutions of creatinine/estimated glomerular filtration rate (eGFR) and cystatin C (CysC), which are markers of glomerular function, as well as urinary N-acetyl-beta-D-glucosaminidase (NAG), kidney injury molecule (KIM)-1, and plasma and urinary neutrophil gelatinase-associated lipocalin (NGAL), which are markers of tubular function. We also aimed to determine if the patient-specific evolutions of these biomarkers can predict (adverse) clinical outcome in patients with clinically stable heart failure.

REFERENCES

1. Townsend N, Wilson L, Bhatnagar P, Wickramasinghe K, Rayner M, Nichols M. Cardiovascular disease in Europe: epidemiological update 2016. Eur Heart J. 2016;37:3232-45.
2. https://healthmetrics.heart.org/wp-content/uploads/2017/10/Projections-of-Cardiovascular-Disease.pdf.
3. https://opendata.cbs.nl.
4. De Boer AR, van Dis I, Visseren FLJ, Vaartjes I, Bots ML. Hart- en vaatziekten in Nederland 2019, cijfers over risicofactoren, hartinterventies, ziekte en sterfte. Den Haag: Hartstichting, 2019.
5. Sianos G, Morel MA, Kappetein AP, Morice MC, Colombo A, Dawkins K, et al. The SYNTAX Score: an angiographic tool grading the complexity of coronary artery disease. EuroIntervention. 2005;1:219-27.
6. Mohr FW, Morice MC, Kappetein AP, Feldman TE, Stahle E, Colombo A, et al. Coronary artery bypass graft surgery versus percutaneous coronary intervention in patients with three-vessel disease and left main coronary disease: 5-year follow-up of the randomised, clinical SYNTAX trial. Lancet. 2013;381:629-38.
7. Garcìa-Garcìa HM1, Gogas BD, Serruys PW, Bruining N. IVUS-based imaging modalities for tissue characterization: similarities and differences. Int J Cardiovasc Imaging. 2011;27:215-24.
8. Waxman S, Dixon SR, L'Allier P, Moses JW, Petersen JL, Cutlip D, et al. In vivo validation of a catheter-based near-infrared spectroscopy system for detection of lipid core coronary plaques: initial RESULTS of the SPECTACL study. JACC Cardiovasc Imaging. 2009;2:858-68.
9. Madder RD, Smith JL, Dixon SR, Goldstein JA. Composition of target lesions by near-infrared spectroscopy in patients with acute coronary syndrome versus stable angina. Circ Cardiovasc Interv. 2012;5:55-61.
10. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002;105:1135-43.
11. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340:115-26.
12. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med. 2008;359(21):2195-207.
13. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur J Heart Fail. 2016;18:891-975.
14. Braunwald E. Biomarkers in heart failure. N Engl J Med. 2008;358:2148-59.
15. Lofman I, Szummer K, Hagerman I, Dahlstrom U, Lund LH, Jernberg T. Prevalence and prognostic impact of kidney disease on heart failure patients. Open Heart. 2016;3(1):e000324.

PART I

Blood biomarkers and novel imaging techniques in coronary artery disease



2

SYNTAX score in relation to intravascular ultrasound and near-infrared spectroscopy for the assessment of atherosclerotic burden in patients with coronary artery disease

2

SYNTAX score in relation to intravascular ultrasound and near-infrared spectroscopy for the assessment of atherosclerotic burden in patients with coronary artery disease

Maxime M. Vroegindewey, Anne-Sophie Schuurman, Isabella Kardys, Sharda S. Anroedh, Rohit M. Oemrawsingh, Jurgen Ligthart, Hector M. Garcia-Garcia, Robert-Jan M. van Geuns, Evelyn Regar, Nicolas M. van Mieghem, Patrick W. Serruys, Eric Boersma, K. Martijn Akkerhuis

*These authors contributed equally.
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ABSTRACT

Aims

To examine the relationship between the anatomical SYNTAX score (SXscore), derived from all three coronary arteries, and coronary wall pathology measured by radiofrequency-intravascular ultrasound (RF-IVUS) and near-infrared spectroscopy (NIRS) in a single non-culprit segment.

Methods and results

In patients referred for coronary angiography (N=88) or PCI (N=592) for stable angina or acute coronary syndrome, the SYNTAX score calculator (www.syntaxscore.com) was used to determine SXscore before PCI, if applicable. RF-IVUS and/or NIRS were performed in a non-stenotic 40 mm study segment following the clinically indicated angiography/PCI. After adjustment for multiple confounders, a higher SXscore was associated with higher segmental plaque volume in the study segment (2.21 mm3 per SXscore point, 95%CI 0.92-3.50, p-value 0.001), as well as with higher volume of fibrous (0.93 mm3 per point) and fibro-fatty tissue (0.29 mm3 per point). A higher SXscore was also associated with a higher NIRS-derived lipid core burden index in the full study segment (1.35 units per SXscore point, 95%CI 0.22-2.47, p-value 0.019). Importantly, SXscore correlated with the fatty/fibro-fatty and LCBI signals despite adjusting for plaque burden.

Conclusions

In patients with CAD, higher SXscores are associated with higher atherosclerotic burden as assessed by RF-IVUS and NIRS in a single non-stenotic coronary artery segment.

INTRODUCTION

The SYNTAX score (SXscore) is an angiographic tool that grades the complexity of coronary artery disease (CAD) and is also used for short- and long-term prediction of major adverse cardiac events (MACE) in patients undergoing percutaneous coronary intervention (PCI) and/or coronary artery bypass graft surgery (CABG).(1-2) The severity and composition of coronary atherosclerosis as assessed by (radiofrequency-)intravascular ultrasound (RF-IVUS) and near-infrared spectroscopy (NIRS) in *one* (non-)stenotic coronary artery segment have recently also shown prognostic value for MACE.(3-8) Furthermore, RF-IVUS and NIRS in one (non-)stenotic coronary artery segment have previously been used to evaluate the effects over time of anti-atherosclerotic therapy under the assumption that these assessments are representative of the total coronary atherosclerotic burden.(9) However, it has never been investigated in a large cohort how well the atherosclerotic burden as graded by NIRS and RF-IVUS measured in one (non-) stenotic coronary artery segment correlates with the atherosclerotic burden as assessed by the SXscore which is derived from all three coronary arteries.

It is important to realize that the three methods differ from each other in the assessment and quantification of CAD. The SXscore is an anatomical scoring tool that grades luminal coronary obstruction, directly from the coronary angiography (CAG). Therefore, it lacks detail with respect to coronary artery wall pathology. Conversely, RF-IVUS and NIRS have been shown to provide information on plaque morphology in the imaged coronary segment. However, both of these imaging techniques require additional intracoronary catheters, whereas the SXscore itself does not require instrumentation of the coronary lumen.

The aim of this study is to examine the relationship between the coronary atherosclerotic burden measured as luminal coronary obstruction graded by the SXscore, derived from all three coronary arteries, and the atherosclerotic burden by assessing coronary artery wall pathology measured by RF-IVUS and NIRS in one non-stenotic segment of a single non-culprit coronary artery.

METHODS

Study population

This study constitutes a combined analysis of two cohorts: The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis-IVUS (ATHEROREMO-IVUS) study and the Integrated Biomarker and Imaging Study-3 (IBIS-3). The design of both studies has been described elsewhere.(9-11) In total, 770 patients with an indication for diagnostic CAG and/or PCI due to either stable angina pectoris (SAP) or an acute coronary syndrome (ACS) were included and had a RF-IVUS and/or NIRS performed in a non-stenotic segment of a non-culprit coronary artery.

Both studies were approved by the Medical Ethics Committee of the Erasmus MC and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all included patients. ATHEROREMO-IVUS is registered in ClinicalTrials.gov, number NCT01789411, and IBIS-3 is registered in The Netherlands trial register, number NTR2872.

Coronary intravascular ultrasound and near-infrared spectroscopy

RF-IVUS and NIRS methods have been described in detail previously. (9-11) For a comprehensive methods section, refer to the *Supplementary Methods*.

SYNTAX Score

The SXscore was calculated (pre-PCI) for every CAG taken at study entry using the SYNTAX Score calculator (www.syntaxscore.com). Details concerning the calculation of the SXscore have been described elsewhere. (1) In brief, the three coronary arteries are divided in 16 segments, each with a corresponding weighting factor. If there is a lesion producing 50% or more luminal obstruction, the weighting factor is added. Moreover, other factors that reflect the severity of the atherosclerotic lesion and the possible difficulty of a percutaneous treatment, for example lesion length and diffuse disease of the vessel, are taken into account. Eventually, all points are summed to obtain the SXscore reflecting the complexity of the CAD of the patient.

As applied in other all-comers and ST-segment elevation myocardial infarction (STEMI) populations, lesions caused by in-stent restenosis were considered as de novo lesions.(12-14) Occlusions in patients presenting with ACS were scored as occlusions of unknown duration, as the analyst was blinded to all other patient information.(15)

In case of a codominant coronary artery circulation, the vessel mainly responsible for the perfusion of the posterior side of the heart was designated as the dominant coronary artery. Last, patients with a pre-existing CABG, whose CAG is unquantifiable using the SXscore, were excluded.

The SXscores were determined by a trained analyst who was blinded with respect to other patient characteristics and clinical outcome.

STATISTICAL ANALYSIS

Categorical variables are presented as numbers and percentages. The distribution of the continuous variables, including RF-IVUS and NIRS parameters, was examined for normality with Kolmogorov-Smirnov tests. Normally-distributed continuous variables are presented as mean ± standard deviation (SD). Non-normally distributed continuous variables are presented as median and interquartile range (IQR). SXscores were categorized into tertiles based on the distribution of the SXscores in the particular group that was being examined. Kruskal-Wallis tests were used for multiple group comparison of continuous variables. Categorical variables were compared using Pearson Chi-square tests or Fisher-Freeman-Halton Exact tests when appropriate.

Linear and logistic regression analyses were applied to evaluate the relation between SXscore (explanatory) and RF-IVUS- and NIRS-derived (dependent) variables. Variables concerning plaque volume were first normalized for the imaged segment length (i.e. normalized plaque volume=plaque volume / imaged segment length*median segment length of study population). In multivariable analyses, age, gender, hypertension, renal impairment, hypercholesterolemia, diabetes mellitus, smoking, indication for CAG, history of PCI, as well as segmental plaque burden were entered as potential confounders/explanatory factors. Thus, the models allow to conclude on the relation between SXscore and the RF-IVUS/NIRS imaging signals, irrespective of the patient's segmental plaque burden. Assumptions underlying linear regression models were evaluated by visual examination of the residuals.

All statistical tests were 2-tailed and p-values <0.05 were considered significant. SPSS, Version 21.0 (IBM Corp., Armonk, NY, USA) was used for all the analyses.

RESULTS

Baseline characteristics

The current study included 680 patients from the combined ATHEROREMO-IVUS and IBIS-3 cohorts (Figure 1). The overall SXscore ranged from 0 to 37.5 with a median of 7 (IQR:3-13) and a mean of 8.6±7.4. Baseline clinical and angiographic variables were stratified according to tertiles reflecting the obtained Sxscores (lowest tertile, ≤4; middle tertile, >4 to ≤10; highest tertile >10, Table 1). The highest tertile comprised more men. As expected, more patients in the higher tertiles exhibited 2-or 3-vessel disease, whereas no significant stenosis or 1-vessel disease was more frequently present in patients with the lowest SXscores. More patients with lower SXscores had previously undergone a PCI.

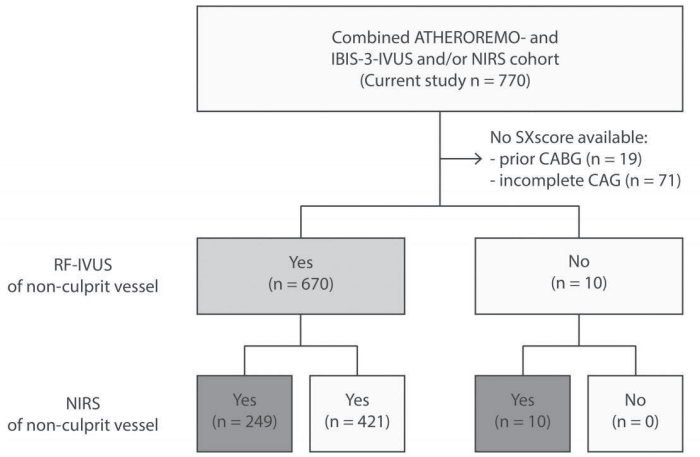


Figure 1. Patient inclusion
RF-IVUS is available in 670 patients(light grey) and NIRS is available in 259 patients(dark grey).
ATHEROREMO-IVUS: The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis-IVUS study, IBIS-3:Integrated Biomarker and Imaging Study-3, NIRS: near-infrared spectroscopy, RF-IVUS: radiofrequency intravascular ultrasound, SXscore: SYNTAX score

Coronary intravascular ultrasound in relation to SXscore

After adjustment for multiple confounders/explanatory factors, a higher SXscore was associated with a higher plaque volume in the study segment (2.21 mm3 per SXscore point, 95%CI 0.92-3.50, p-value 0.001) (Table 2). The relation between SXscore and plaque burden was consistent with this observation, although statistically non-significant (p-value 0.078). A higher SXscore was also associated with a

Table 1. Baseline characteristics

	Low SXscore	Mid SXscore	High SXscore	p-value
	≤4 (n=236)	>4 to ≤10 (n=221)	>10 (n=223)	
Patient characteristics				
Age, years	60.4±10.9	61.3±10.7	61.2±11.0	0.70
Men, n(%)	169(71.6)	169(76.5)	184(82.5)	0.022
Risk factors				
Diabetes Mellitus, n(%)	48(20.3)	42(19.0)	40(17.9)	0.79
Hypertension, n(%)	130(55.1)	130(58.8)	107(48.0)	0.064
Hypercholesterolemia, n(%)	119(50.4)	138(62.4)	118(52.9)	0.028
Smoking, n(%)	63(26.7)	66(29.9)	70(31.4)	0.55
History				
Positive family history, n(%)	138(58.5)	117(52.9)	101(45.3)	0.018
Previous myocardial infarction, n(%)	79(33.5)	59(26.7)	61(27.4)	0.21
Previous PCI, n(%)	94(39.8)	63(28.5)	52(23.3)	<0.001
Previous stroke, n(%)	15(6.4)	12(5.4)	15(6.7)	0.84
History of peripheral artery disease, n(%)	12(5.1)	19(8.6)	14(6.3)	0.31
History of renal insufficiency, n(%)	13(5.5)	10(4.5)	11(4.9)	0.89
History of heart failure, n(%)	8(3.4)	4(1.8)	5(2.2)	0.56
Procedural characteristics				
Indication for coronary angiography				0.001
Stable angina, n(%)	130(55.1)	95(43.0)	88(39.5)	
Acute coronary syndromes, n(%)	106(44.9)	126(57.0)	135(60.5)	
Extent of coronary artery disease				<0.001
No significant stenosis, n(%)	91(38.6)	1(0.5)	0(0.0)	
1-vessel disease, n(%)	134(56.8)	138(62.4)	72(32.3)	
2-vessel disease, n(%)	11(4.7)	69(31.2)	114(51.1)	
3-vessel disease, n(%)	0(0.0)	13(5.9)	37(16.6)	
Imaged coronary artery characteristics				
Imaged segment length, mm	44.7±14.1	42.6±13.1	44.4±14.7	0.17
Imaged coronary artery for RF-IVUS				0.004
Left anterior descending, n(%)	107(46.7)	76(34.5)	64(28.8)	
Left circumflex, n(%)	58(25.1)	78(35.6)	81(36.5)	
Right coronary artery, n(%)	65(28.1)	65(29.9)	76(34.6)	
Imaged coronary artery for NIRS				0.003
Left anterior descending, n(%)	40(44.4)	34(39.1)	20(24.4)	
Left circumflex, n(%)	22(24.4)	36(41.4)	31(37.8)	
Right coronary artery, n(%)	28(31.1)	17(19.5)	31(37.8)	

CABG: coronary artery bypass graft, IQR: interquartile range, LCBI: Lipid Core Burden Index, NIRS: near-infrared spectroscopy, PCI: percutaneous intervention, RF-IVUS:(radiofrequency) intravascular ultrasound, SXscore: SYNTAX score

higher volume of fibrous (0.93 mm3 per SXscore point, 95%CI 0.53-1.33, p-value <0.001) and fibro-fatty tissue (0.29 mm3 per SXscore point, 95% CI 0.17-0.42, p-value <0.001) (Tables 2, Figure 2). Importantly, the SXscore correlated with the fatty/fibro-fatty signals despite adjusting for plaque burden. In contrast, we found no association between SXscore and necrotic core volume (p-value 0.16) or the presence of TCFA (p-value 0.46).

Table 2. Associations between the SYNTAX score and RF-IVUS and NIRS derived variables in multivariable analyses

RF-IVUS derived variables	Mean/OR (95%CI)						β/OR† (95% CI)		p-value‡
	SXscore ≤ 4		4 < SXscore ≤ 10		Sxscore > 10				
No. of patients	230		219		221		670		
Plaque volume, mm^3 ‡	230.1	(215.7-244.4)	246.0	(222.3-269.6)	242.4	(228.8-276.1)	2.21	(0.92-3.50)	0.001
Plaque burden, %‡	38.1	(36.8-39.5)	38.8	(36.7-40.9)	39.0	(37.0-41.1)	0.10	(-0.011-0.22)	0.078
Plaque composition									
Fibrous, mm^3	61.4	(55.1-67.7)	68.0	(60.7-75.4)	72.6	(65.3-80.2)	0.93	(0.53-1.33)	<0.001
Fibro-fatty, mm^3	10.8	(9.3-12.3)	13.6	(11.3-15.9)	14.9	(12.6-17.2)	0.29	(0.17-0.42)	<0.001
Dense calcium, mm^3	13.3	(11.3-15.3)	14.3	(11.9-16.8)	13.9	(11.4-16.3)	0.023	(-0.11-0.16)	0.73
Necrotic core, mm^3	26.1	(22.8-29.3)	29.2	(25.7-32.6)	27.5	(24.0-30.9)	0.14	(-0.52-0.33)	0.16
Lesion morphology									
TCFA	1		0.97	(0.63-1.50)	0.81	(0.53-1.24)	0.99	(0.97-1.01)	0.46
MLA ≤4.0mm^2 ‡	1		0.74	(0.44-1.23)	0.95	(0.59-1.54)	1.00	(0.97-1.02)	0.80
Plaque burden ≥70%‡	1		1.05	(0.58-1.88)	1.47	(0.85-2.53)	1.02	(1.00-1.05)	0.092
NIRS-derived variables	SXscore ≤ 3		3 < SXscore ≤ 8		SXscore > 8				
No. of patients	90		87		82		259		
LCBI region of interest	39.4	(27.9-50.8)	56.0	(36.2-75.9)	62.7	(42.6-82.9)	1.35	(0.22-2.47)	0.019
LCBI worst 10 mm	118.1	(90.9-145.3)	150.3	(106.4-194.3)	176.9	(132.2-221.6)	2.89	(0.39-5.38)	0.024
LCBI worst 4 mm	190.4	(154.6-226.2)	231.3	(175.7-286.5)	266.6	(210.2-323.1)	3.83	(0.69-6.97)	0.017

We present means and odds ratios with 95%CI based on multivariable models with SX score included as categorical (explanatory) variable. In addition, we present β's and odds ratios with SXscore included as continuous (explanatory) variable. Multivariable models are adjusted for age, gender, hypertension, renal impairment, hypercholesterolemia, diabetes mellitus, smoking, indication for CAG, history of PCI and plaque burden.

† Based on multivariable models with SXscore included as continuous (explanatory) variable

‡ Multivariable model without adjustment for plaque burden

CI: confidence interval, LCBI: lipid core burden index, MLA: minimum luminal area, NIRS: near-infrared spectroscopy, No: number, OR: odds ratio, RF-IVUS: radiofrequency intravascular ultrasound, SXscore: SYNTAX score, TCFA: thin-cap fibroatheromas

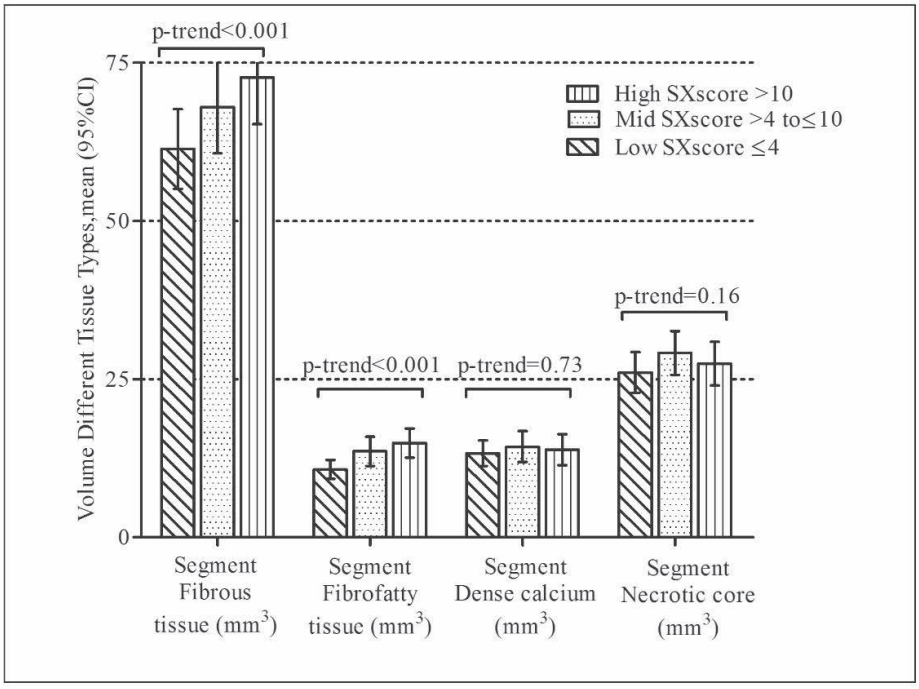


Figure 2. Distribution of RF-IVUS derived plaque components across the SXscore categorized in tertiles

The mean volumes of the different plaque components: fibrous, fibro-fatty, dense calcium and necrotic tissue are divided across the SXscore categorized in tertiles (cut-off points 4 and 10).

RF-IVUS: radiofrequency intravascular ultrasound, SXscore: SYNTAX score

Near-infrared spectroscopy in relation to SXscore

A higher SXscore was associated with a higher NIRS-derived lipid core burden index (LCBI) in the full study segment (1.35 units per SXscore point, 95%CI 0.22-2.47, p-value 0.019) (Tables 2, Figure 3). Consistent results were observed for the 10- and 4-mm segments with highest LCBI values. Again, it is relevant to note that the observed correlation between SXscore and LCBI signals was independent of segment plaque burden.

DISCUSSION

This is the first study, to our knowledge, that systematically examined a large patient population for the correlation of coronary atherosclerotic burden as determined by the SXscore and the extent and characteristics of coronary atherosclerosis as

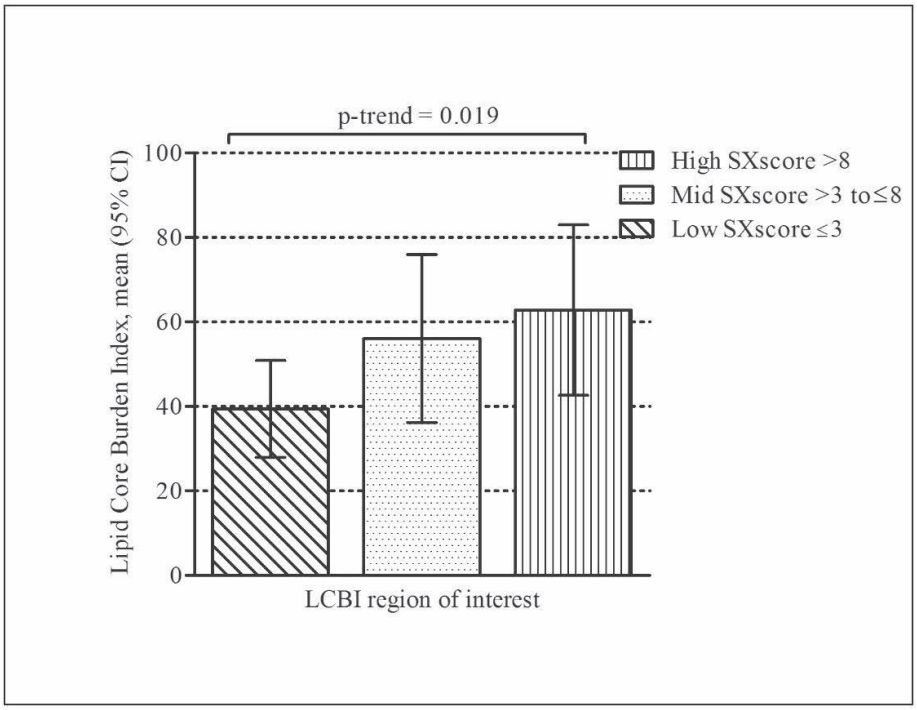


Figure 3. Distribution of NIRS-derived LCBI across the SXscore categorized in tertiles
The mean LCBI of the region of interest is divided across the SXscore categorized in tertiles (cut-off points 3 and 8).
LCBI: lipid core burden index, SXscore: SYNTAX score

assessed by RF-IVUS and NIRS in one non-stenotic segment of a single non-culprit coronary artery. This study shows that there is a significant and independent association between these entities in patients with CAD.

The SXscore is a well-established angiographic tool for the assessment of the severity and complexity of CAD. (2) It not only evaluates the number of significant stenoses but also lesion length and amount of calcification, amongst others. Still, as the SXscore is based on coronary luminography, it is limited in the assessment of the extent of (non-stenotic) plaque burden and plaque morphology, including the identification of high-risk plaque characteristics and vulnerable plaques. We demonstrated that the SXscore is associated with RF-IVUS and NIRS derived information on the extent and composition of coronary atherosclerosis in patients with CAD. The correlation between SXscore and the amount of fatty/fibro-fatty tissue as well as LCBI were most striking. In this respect it is relevant to note the absence of

relations between SXscore and plaque phenotype (necrotic core volume) and lesion morphology (TCFA).

Previously, a significant relation between atherosclerotic burden in one non-culprit coronary segment as assessed by RF-IVUS or NIRS and cardiovascular outcome was demonstrated which persisted after exclusion of culprit-related and imaged segment-related cardiac events.(5,7) This indirectly supported the assumption that the atherosclerotic burden in one non-culprit coronary segment may be representative for the atherosclerotic disease of the entire coronary tree. The current study shows a direct association between angiographic atheroma burden of all three vessels and intravascular coronary wall evaluation of a non-culprit segment.

Although pre-specified high-risk plaque phenotypes (TCFA, MLA≤4.0mm^2 and lesions with a plaque burden of ≥70%) were not significantly associated with an increase in SXscore, the volume of fibrous and fibro-fatty tissue in plaques was higher in patients with a higher SXscore. Although a previous study has shown that plaque morphology, as measured by three-vessel imaging by optical coherence tomography (OCT) or IVUS, is associated with and may be used for the identification of vulnerable plaques in patients with ACS,(19) it appears from our study that it is the amount of tissue type which is associated with SXscore and not plaque morphology (the layout of the tissue) per se. In light of the relatively overall low angiographic burden of disease in our population, however, it needs to be considered that this finding may not be applicable in a patient population with more advanced CAD. Moreover, necrotic core and dense calcium did not show a significant association with a higher SXscore.

Previously, in one other small cohort, the relationship between NIRS and SXscore was explored but no association was found.(17) The relationship between NIRS and the SXscore has also been studied in a subset of patients from ATHEROREMO-IVUS.(18) The enrichment of the ATHEROREMO-IVUS cohort with the IBIS-3 cohort in the current study substantially increases the sample size and creates more robust data.

In most studies, SXscore is stratified in tertiles or even quartiles reflecting the distribution of the scores found in the respective cohort.(2) The thresholds of the original SYNTAX trial (cut-off points:22 and 33) have been incorporated in the guidelines for the decision-making regarding CABG and PCI, but these thresholds apply to patients with left main and/or three-vessel disease.(19) Our population also consisted of patients with single or two vessel disease and hence, understandably, our mean SXscore and cut-off values for the tertiles were relatively low. It warrants further research to assess which absolute SXscore thresholds are applicable in a

heterogeneous population for risk prediction of adverse outcome in patients with CAD.

Furthermore, we argue that combined IVUS-NIRS intracoronary imaging holds promise for more precise detection and quantification of atherosclerotic burden in patients with CAD, and in the future may even be of interest for the prediction of adverse events. However, further research is warranted to assess the application of combined IVUS-NIRS intracoronary imaging for the prediction of adverse events.

Limitations

This cohort, composed of two prospective studies, has broad inclusion criteria which enable the results to be applicable in a broad patient population with CAD. Data collection, processing and analyses were conducted by researchers independent and blinded for patient and outcome data. However, a few limitations deserve consideration.

As indicated, our study includes patients with relatively low SXscores. This might induce an underestimation of the studied associations and insufficient power to reveal additional associations. However, a subgroup analysis with exclusion of patients without significant CAD, showed results that were essentially similar. Moreover, the lowest tertile in this cohort contains significantly more patient with a previous PCI, which may indicate an underestimation of the severity of CAD caused by a low SXscore derived at study entry.

Furthermore, while the SXscore analyst was blinded for all patient information, occlusions in STEMI patients were scored as occlusions of unknown duration. In the MI SYNTAX score study, it was suggested to calculate occlusions in STEMI patients post-wiring. (20) However, the MI SYNTAX score did not show better performance than the original SXscore calculated in STEMI patients.

Lastly, although literature demonstrated that experienced operators produce reasonable SXscores, the modest reproducibility of the SXscore in general has to be acknowledged. (21) However, because of the overall relatively low angiographic burden of disease in our study population, we expected a fair reproducibility of the SXscore in our study. To address the reproducibility of our SXscores, a second experienced operator, blinded for patient characteristics and previously scored SXscores, repeated SXscore analysis in a representative random sample. Cohen's kappa showed to be 0.91, indicating a good interobserver agreement.

Conclusions

In patients with CAD, there is a clear and significant correlation between a higher SXscore and a higher atherosclerotic burden as assessed by RF-IVUS and NIRS in one non-stenotic segment in a single non-culprit coronary artery.

REFERENCES

1. Sianos G, Morel MA, Kappetein AP, Morice MC, Colombo A, Dawkins K, van den Brand M, Van Dyck N, Russell ME, Mohr FW, Serruys PW. The SYNTAX Score: an angiographic tool grading the complexity of coronary artery disease. EuroIntervention. 2005; 1:219-27.
2. Mohr FW, Morice MC, Kappetein AP, Feldman TE, Stahle E, Colombo A, Mack MJ, Holmes DR Jr, Morel MA, Van Dyck N, Houle VM, Dawkins KD, Serruys PW. Coronary artery bypass graft surgery versus percutaneous coronary intervention in patients with three-vessel disease and left main coronary disease: 5-year follow-up of the randomised, clinical SYNTAX trial. Lancet. 2013;381:629-38.
3. Garcia-Garcia HM, Mintz GS, Lerman A, Vince DG, Margolis MP, van Es GA, Morel MA, Nair A, Virmani R, Burke AP, Stone GW, Serruys PW. Tissue characterisation using intravascular radiofrequency data analysis: recommendations for acquisition, analysis, interpretation and reporting. EuroIntervention. 2009;5: 177-89.
4. Waxman S, Dixon SR, L'Allier P, Moses JW, Petersen JL, Cutlip D, Tardif JC, Nesto RW, Muller JE, Hendricks MJ, Sum ST, Gardner CM, Goldstein JA, Stone GW, Krucoff MW. In vivo vali¬dation of a catheter-based near-infrared spectroscopy system for detection of lipid core coronary plaques: initial results of the SPECTACL study. JACC Cardiovasc Imaging. 2009;2:858-68.
5. Oemrawsingh RM, Cheng JM, Garcia-Garcia HM, van Geuns RJ, de Boer SP, Simsek C, Kardys I, Lenzen MJ, van Domburg RT, Regar E, Serruys PW, Akkerhuis KM, Boersma E; ATHEROREMO-NIRS Investigators. Near-infrared spectroscopy predicts cardiovascular outcome in patients with coronary artery disease. J Am Coll Cardiol. 2014;64:2510-8.
6. Nicholls SJ, Hsu A, Wolski K, Hu B, Bayturan O, Lavoie A, Uno K, Tuzcu EM, Nissen SE. Intravascular ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol. 2010;55:2399-407.
7. Cheng JM, Garcia-Garcia HM, de Boer SP, Kardys I, Heo JH, Akkerhuis KM, Oemrawsingh RM, van Domburg RT, Ligthart J, Witberg KT, Regar E, Serruys PW, van Geuns RJ, Boersma E. 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.
8. Schuurman AS, Vroegindewey M, Kardys I, Oemrawsingh RM, Cheng JM, de Boer S, Garcia-Garcia HM, van Geuns RJ, Regar ES, Daemen J, van Mieghem NM, Serruys PW, Boersma E, Akkerhuis KM. Near-infrared spectroscopy-derived lipid core burden index predicts adverse cardiovascular outcome in patients with coronary artery disease during long-term follow-up. Eur Heart J. 2018;39:295-302.
9. Oemrawsingh RM, Garcia-Garcia HM, van Geuns RJ, Lenzen MJ, Simsek C, de Boer SP, Van Mieghem NM, Regar E, de Jaegere PP, Akkerhuis KM, Ligthart JM, Zijlstra F, Serruys PW, Boersma E. Integrated Biomarker and Imaging Study 3 (IBIS-3) to assess the ability of rosuvastatin to decrease necrotic core in coro¬nary arteries. EuroIntervention. 2016;12:734-9.
10. Simsek C, Garcia-Garcia HM, van Geuns RJ, Magro M, Girasis C, van Mieghem N, Lenzen M, de Boer S, Regar E, van der Giessen W, Raichlen J, Duckers HJ, Zijlstra F, van der Steen T, Boersma E, Serruys PW; Integrated Biomarker and Imaging Study-3 investigators. The ability of high dose rosuvastatin to improve plaque composition in

non-intervened coronary arteries: rationale and design of the Integrated Biomarker and Imaging Study-3 (IBIS-3). EuroIntervention. 2012;8:235-41.

11. de Boer SP, Cheng JM, Garcia-Garcia HM, Oemrawsingh RM, van Geuns RJ, Regar E, Zijlstra F, Laaksonen R, Halperin E, Kleber ME, Koenig W, Boersma E, Serruys PW. Relation of genetic profile and novel circulating biomarkers with coronary plaque phe¬notype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. EuroIntervention. 2014;10:953-60.
12. Wykrzykowska JJ, Garg S, Girasis C, de Vries T, Morel MA, van Es GA, Buszman P, Linke A, Ischinger T, Klauss V, Corti R, Eberli F, Wijns W, Morice MC, di Mario C, van Geuns RJ, Juni P, Windecker S, Serruys PW. Value of the SYNTAX score for risk assessment in the all-comers population of the randomized multi¬center LEADERS (Limus Eluted from A Durable versus ERodable Stent coating) trial. J Am Coll Cardiol. 2010;56:272-7.
13. Girasis C, Garg S, Räber L, Sarno G, Morel MA, Garcia- Garcia HM, Lüscher TF, Serruys PW, Windecker S. SYNTAX score and Clinical SYNTAX score as predictors of very long-term clinical outcomes in patients undergoing percutaneous coronary interventions: a substudy of SIRolimus-eluting stent compared with pacliTAXel-eluting stent for coronary revascularization (SIRTAX) trial. Eur Heart J. 2011;32:3115-27.
14. Garg S, Serruys PW, Silber S, Wykrzykowska J, van Geuns RJ, Richardt G, Buszman PE, Kelbaek H, van Boven AJ, Hofma SH, Linke A, Klauss V, Wijns W, Macaya C, Garot P, DiMario C, Manoharan G, Kornowski R, Ischinger T, Bartorelli A, Van Remortel E, Ronden J, Windecker S. The prognostic utility of the SYNTAX score on 1-year outcomes after revascularization with zotarolimus- and everolimus-eluting stents: a substudy of the RESOLUTE All Comers Trial. JACC Cardiovasc Interv. 2011;4: 432-41.
15. Garg S, Sarno G, Serruys PW, Rodriguez AE, Bolognese L, Anselmi M, De Cesare N, Colangelo S, Moreno R, Gambetti S, Monti M, Bristot L, Bressers M, Garcia-Garcia HM, Parrinello G, Campo G, Valgimigli M; STRATEGY and MULTISTRATEGY Investigators. Prediction of 1-year clinical outcomes using the SYNTAX score in patients with acute ST-segment elevation myo¬cardial infarction undergoing primary percutaneous coronary intervention: a substudy of the STRATEGY (Single High-Dose Bolus Tirofiban and Sirolimus-Eluting Stent Versus Abciximab and Bare-Metal Stent in Acute Myocardial Infarction) and MULTISTRATEGY (Multicenter Evaluation of Single High-Dose Bolus Tirofiban Versus Abciximab With Sirolimus-Eluting Stent or Bare-Metal Stent in Acute Myocardial Infarction Study) trials. JACC Cardiovasc Interv. 2011;4:66-75.
16. Tian J, Ren X, Vergallo R, Xing L, Yu H, Jia H, Soeda T, McNulty I, Hu S, Lee H, Yu B, Jang IK. Distinct morphological features of ruptured culprit plaque for acute coronary events com¬pared to those with silent rupture and thin-cap fibroatheroma: a combined optical coherence tomography and intravascular ultra¬sound study. J Am Coll Cardiol. 2014;63:2209-16.
17. Zynda TK, Thompson CD, Hoang KC, Seto AH, Glovaci D, Wong ND, Patel PM, Kern MJ. Disparity between angiographic coronary lesion complexity and lipid core plaques assessed by near-infrared spectroscopy. Catheter Cardiovasc Interv. 2013;81: 529-37.
18. Brugaletta S, Magro M, Simsek C, Heo JH, de Boer S, Ligthart J, Witberg K, Farooq V, van Geuns RJ, Schultz C, van Mieghem N, Regar E, Zijlstra F, Duckers HJ, de Jaegere P, Muller JE, van der Steen AF, Boersma E, Garcia-Garcia HM, Serruys PW. Plaque compositional Syntax score: combining angio-graphy and lipid burden in coronary artery disease. JACC Cardiovasc Imaging. 2012;5(3 Suppl):S119-21.

19. Authors/Task Force members, Windecker S, Kolh P, Alfonso F, Collet JP, Cremer J, Falk V, Filippatos G, Hamm C, Head SJ, Jüni P, Kappetein AP, Kastrati A, Knuuti J, Landmesser U, Laufer G, Neumann FJ, Richter DJ, Schauerte P, Sousa Uva M, Stefanini GG, Taggart DP, Torracca L, Valgimigli M, Wijns W, Witkowski A. 2014 ESC/EACTS Guide¬lines on myocardial revascularization: The Task Force on Myo¬cardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Sur¬gery (EACTS)Developed with the special contribution of the European Association of Percutaneous Cardiovascular Interven¬tions (EAPCI). Eur Heart J. 2014;35: 2541-619.
20. Magro M, Nauta S, Simsek C, Onuma Y, Garg S, van der Heide E, van der Giessen WJ, Boersma E, van Domburg RT, van Geuns RJ, Serruys PW. Value of the SYNTAX score in patients treated by primary percutaneous coronary intervention for acute ST-elevation myocardial infarction: The MI SYNTAXscore study. Am Heart J. 2011;161:771-81.
21. Garg S, Girasis C, Sarno G, Goedhart D, Morel MA, Garcia- Garcia HM, Bressers M, van Es GA, Serruys PW; SYNTAX trial investigators. The SYNTAX score revisited: a reassessment of the SYNTAX score reproducibility. Catheter Cardiovasc Interv. 2010;75:946-52.
22. Nair A, Margolis MP, Kuban BD, Vince DG. Automated coronary plaque characterisation with intravascular ultrasound backscatter: ex vivo validation. EuroIntervention. 2007;3: 113-20.
23. Rodriguez-Granillo GA, Garcia-Garcia HM, Mc Fadden EP, Valgimigli M, Aoki J, de Feyter P, Serruys PW. In vivo intravascu¬lar ultrasound-derived thin-cap fibroatheroma detection using ultra¬sound radiofrequency data analysis. J Am Coll Cardiol. 2005;46: 2038-42.
24. Erlinge D. Near-infrared spectroscopy for intracoronary detection of lipid-rich plaques to understand atherosclerotic plaque biology in man and guide clinical therapy. J Intern Med. 2015; 278:110-25.

SUPPLEMENTARY APPENDIX

METHODS

Coronary intravascular ultrasound

Following CAG, IVUS was performed in a proximal non-stenotic (<50% stenosis) segment of at least 40 mm of a non-culprit artery. The order of preference used for selection of the non-culprit vessel was predefined in the study protocol: 1) left anterior descending artery; 2) right coronary artery; 3) left circumflex artery. All IVUS data were obtained 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 pullback speed of 0.5 mm per second. The baseline IVUS images were sent to an independent core laboratory (Cardialysis, Rotterdam, the Netherlands) for offline analysis. The core laboratory personnel were blinded for baseline patient characteristics and clinical outcome. The RF-IVUS analysis was performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, CA, USA) software.

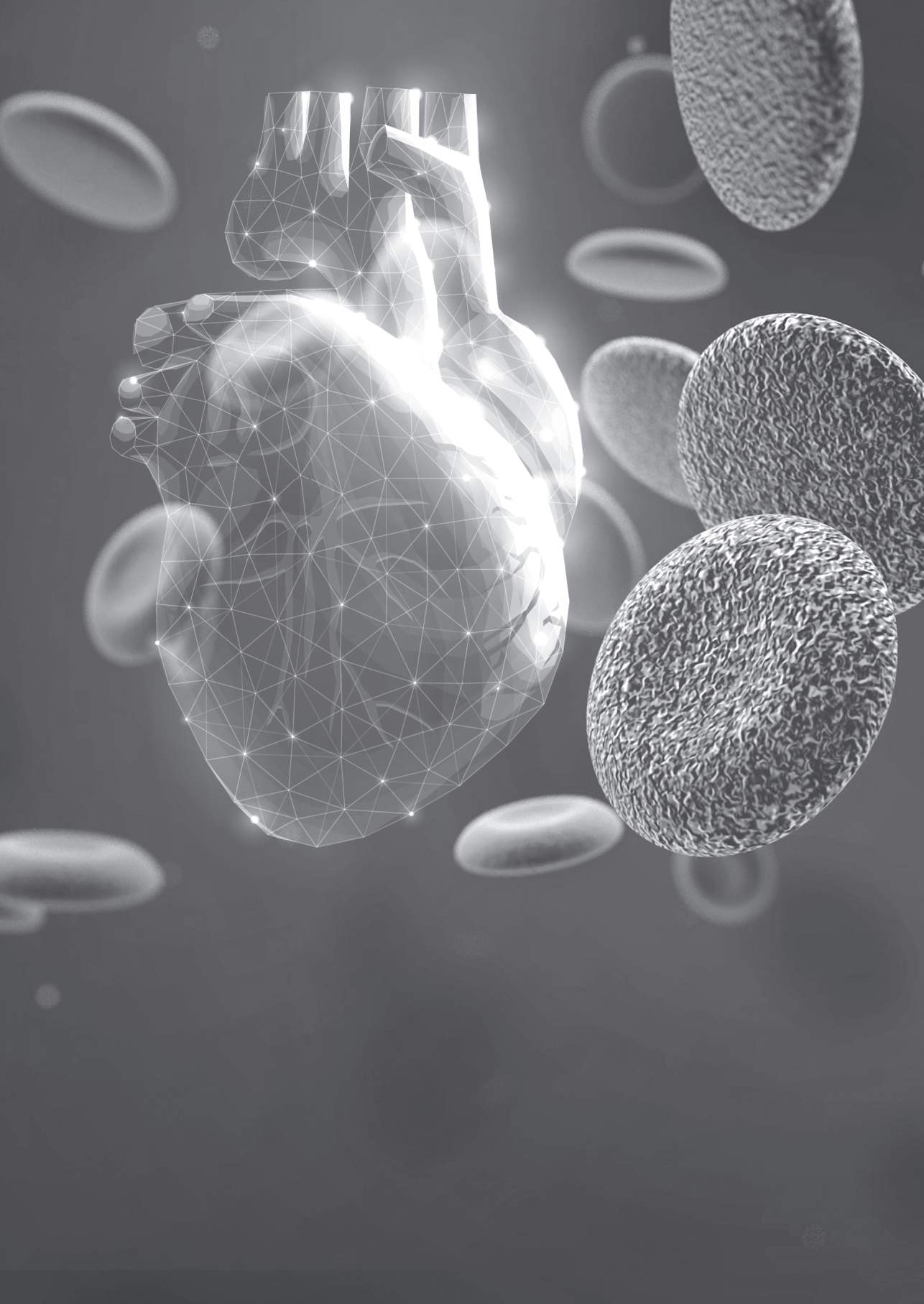
The external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40 mm). Plaque burden was defined as the plaque and media cross-sectional area divided by the external elastic membrane cross-sectional area. A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least 3 consecutive frames. The composition of atherosclerotic plaque was characterized into 4 different tissue types: fibrous, fibro-fatty, dense calcium and necrotic core [22]. Three types of high-risk lesions were identified: 1) 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 (MLA) of ≤4.0 mm^2. (4,23)

Methods near-infrared spectroscopy

In a subset of patients, NIRS imaging was performed in the same segment as IVUS. The NIRS system used consists of a 3.2 Fr rapid exchange catheter, a pullback and rotation device and a console (Infraredx, Burlington, MA, USA), approved by the U.S. Food and Drug Administration. Image acquisition was performed by a motorized catheter pullback at a speed of 0.5 mm/s and 240 rpm. The system performed 1,000 chemical measurements per 12.5 mm. Each measurement interrogated 1 to

2 mm^2 of vessel wall from, approximately, 1 mm in depth from the luminal surface towards the adventitia.(4,5)

The NIRS measurements were used to create a chemogram. The fraction of yellow pixels from the chemogram was multiplied by 1,000, to calculate the lipid core burden index (LCBI). Thus, the LCBI value, with a range between 0 and 1,000, represents the amount of lipid core in the assessed segment. (24) In addition, within this region of interest, the 10 mm and 4 mm segment with the highest LCBI was defined. NIRS images were analyzed offline by an independent core laboratory (Cardialysis, Rotterdam, the Netherlands). Core laboratory personnel were blinded to all other patient and outcome data.



3

Associations of 26 Circulating Inflammatory and Renal Biomarkers with Near-Infrared Spectroscopy and Long-term Cardiovascular Outcome in Patients Undergoing Coronary Angiography (ATHEROREMO-NIRS Substudy)

3

Associations of 26 Circulating Inflammatory and Renal Biomarkers with Near-Infrared Spectroscopy and Long-term Cardiovascular Outcome in Patients Undergoing Coronary Angiography (ATHEROREMO-NIRS Substudy)

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ABSTRACT

Purpose of review: The purpose of this study was to investigate the association of 26 inflammatory biomarkers (acute phase proteins, cytokines, chemokines) and renal markers with coronary lipid core burden index (LCBI) assessed by near-infrared spectroscopy (NIRS) imaging, as well as the association of these biomarkers with long-term cardiovascular outcome.

Recent findings: NIRS-derived LCBI has recently been shown to be an independent predictor of major adverse cardiac events (MACE). However, studies on the association between circulating biomarkers and NIRS-derived characteristics have not yet been performed.

Summary: Between 2008 and 2011, 581 patients underwent diagnostic coronary angiography or percutaneous coronary intervention for stable angina pectoris or acute coronary syndrome (ACS). NIRS of a non-culprit vessel was performed in a subset of 203 patients. In multivariable analyses, TNF-α tended to be associated with higher LCBI (Beta: 0.088 ln(pg/ml) increase per unit LCBI; 95% CI: 0.000-0.177, p=0.05) after adjustment for clinical characteristics. However, this association did not persist after Bonferroni correction (statistical threshold: 0.0019). Major adverse cardiac events (MACE) were registered in 581 patients during a median follow-up time of 4.7 years (IQR: [4.2-5.6] years). After adjustment for clinical characteristics and Bonferroni correction, IL-8 (HR: 1.60; 95%CI[1.18-2.17]per ln(pg/ml), p=0.002) was borderline associated with MACE and significantly associated with all-cause mortality or ACS (HR: 1.75; 95%CI[1.24-2.48]per ln(pg/ml), p=0.0015).

In conclusion, we found that IL-8 was independently associated with clinical outcome, but altogether, the multiplex panel we investigated here did not render a useful blood biomarker of high LCBI.

INTRODUCTION

Vulnerable plaque, defined as a plaque that is sensitive to rupture [1], is characterized by a large lipid core, thin fibrous cap and active inflammation [2]. Pathology studies have shown that approximately 60% of acute coronary syndromes (ACS) are caused by ruptures of such vulnerable plaques [3]. Near-infrared spectroscopy (NIRS) is a novel catheter-based imaging technique based on diffuse reflectance spectroscopy [4]. This technique is capable of characterizing the chemical components of the atherosclerotic plaque and is consequently able to identify lipid core [5]. Lipid core plaques (LCP) have been shown to be more vulnerable to rupture than non-LCP [6, 7]. A strong association has been demonstrated between LCP, as detected by NIRS, and cardiovascular events [6].

An alternative, non-invasive way to detect LCP could aid in risk stratification. Blood biomarkers may carry potential to detect vulnerable plaques in an early stage and in a non-invasive manner. Among others, biomarkers of inflammation (such as acute phase proteins, cytokines, and chemokines) and renal markers have strongly been implicated in the atherosclerotic process and in the occurrence of coronary events [8-11, 7, 12]. Currently, to the best of our knowledge, there are no data available on the associations between circulating biomarkers and NIRS measurements**.** Such an investigation could lead to further pathophysiological insights concerning plaque vulnerability, and could help bridge the gap between known biological pathways and clinical imaging findings.

Therefore, the purpose of this study was to investigate the association of 26 circulating inflammatory and renal biomarkers with coronary lipid core burden index (LCBI) as determined in-vivo by NIRS imaging in patients undergoing coronary angiography. Furthermore, the long-term prognostic value of these biomarkers for the occurrence of major adverse cardiac events (MACE) was evaluated.

METHODS

Study population and design

The design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis–intravascular Ultrasound (ATHEROREMO-IVUS), and its sub-study The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis–Near-Infrared Spectroscopy (ATHEROREMO-NIRS), has been described elsewhere [13, 6]. In brief, from 2008 until 2011, 768 patients with an indication for diagnostic coronary angiography (DCO) or percutaneous coronary intervention (PCI) due to stable angina pectoris (SAP) or ACS were included in a biomarker study in Erasmus MC, Rotterdam, the Netherlands. In 581 of these patients, IVUS of a non-culprit vessel was performed (Figure-1). Among these patients, NIRS of the same segment was performed in a subset of 191 patients. In 12 additional patients only NIRS, not IVUS, was performed, rendering a total of 203 patients in whom NIRS measurements were available.

Both studies were approved by the medical ethics committee of the Erasmus MC and were performed in accordance with the criteria described in the declaration of Helsinki. Written informed consent was obtained from all included patients.

Biomarker measurements

Blood samples were drawn from the arterial sheath prior to the diagnostic coronary angiography or PCI procedure and 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 at Erasmus MC in serum using an immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Cobas 8000 modular analyzer platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland). Frozen EDTA-plasma samples were transported under controlled conditions (at a temperature of −80 °C) to Myriad RBM, Austin, Texas, USA, where the concentrations of cytokines, chemokines, acute phase proteins and renal biomarkers were determined using a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, Texas, USA). CRP, Ferritin, Haptoglobin, Plasminogen Activator Inhibitor 1 (PAI 1), fibrinogen, Macrophage Inflammatory Protein-1 alpha (MIP-1 α), Macrophage Inflammatory Protein-1 beta (MIP-1 β), Monocyte Chemotactic Protein 1 (MCP-1), Regulated upon Activation Normal T cell Expressed and Secreted (T-Cell Specific Rantes), Tumor Necrosis Factor Receptor 2(TNF R2), Interleukin-6 (IL-6), Interleukin -8 (IL-8), creatinine, cystatin C, adiponectin and myoglobin were determined in 570 patients from the ATHEROREMO-IVUS population (n=581) and 190 patients of the

ATHEROREMO-NIRS population (n=203) (Supplemental figure 1). Alpha-1-antitrypsin (AAT), alpha-2-macroglobulin (A2Macro), complement C 3 (C3), Tumor Necrosis Factor alpha (TNF-α), Tumor Necrosis Factor beta (TNF-β), Interferon γ (INF-γ), Interleukin-10(IL-10), Interleukin-18 (IL-18), Neutrophil Gelatinase-Associated Lipocalin (NGAL) and beta-2-microglobulin (B2M) were determined in random subsets of 473 and 156 patients, respectively. 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. The biomarker laboratories had no knowledge of clinical or intracoronary imaging data.

Near-infrared spectroscopy

The NIRS coronary imaging system consisted of a 3.2-F rapid exchange catheter, a pullback and rotation device, and a console (InfraReDx, Burlington, Massachusetts, USA). This NIRS system was approved by the U.S. Food and Drug Administration. The NIRS image acquisition was performed in a non-culprit vessel. The order of preference for selection of the non-culprit vessels was predefined in the study protocol: 1) left anterior descending artery; 2) right coronary artery; and 3) left circumflex artery. The NIRS target segment of the non-culprit vessel was required to be at least 40 mm in length and without significant luminal narrowing (<50% stenosis) as assessed by online angiography. Image acquisition was performed by a motorized catheter pullback at a speed of 0.5 mm/s and 240 rpm in a proximal segment of the artery, starting distal to a side branch. Immediately after a pullback, the data in the scanned coronary arterial segment were displayed in a chemogram. The probability of the presence of LCP in the scanned coronary arterial segment was calculated by means of a prediction algorithm and was displayed using colors, ranging from red (low probability of LCP) to yellow-coded plaque (high probability of LCP) [14] (Figure-2). The x-axis of the chemogram represents the pullback position in millimeters and the y-axis the degree of rotation within the artery from 0 to 360 degrees. The block chemogram summarizes the chemogram in 2 mm increments. The numeric value of each block in the block chemogram is the 90^{th} percentile of all pixel values in the corresponding 2-mm chemogram segment.[15]. The block chemogram is mapped to the same color scale as the chemogram, but the display is binned to 4 discrete colors to aid in visual interpretation (red: $P<0.57$, orange: $0.57<P\leq0.84$, tan: $0.84<P\leq0.98$, and yellow: $P>0.98$, with P being the algorithm probability that a LCP is present in that 2-mm block). [15]. The LCBI quantifies the amount of LCP in the entire scanned artery segment on the block chemogram, and is computed

as the fraction of valid pixels that exceed an LCP probability of 0.6, multiplied with 1000 [15]. NIRS images were evaluated offline by an independent core research laboratory (Cardialysis BV, Rotterdam, the Netherlands) that had no knowledge of any other patient, biomarker or outcome data.

Follow-up and study endpoints

Clinical and vital status of patients were collected from medical charts, civil registries or by written or telephone contact with the patients or relatives. Specifically, all living patients participating in the IVUS/NIRS study were systematically questioned on the occurrence of MACE and re-admission. For patients with adverse events, hospital discharge letters were obtained and treating physicians or hospitals were contacted if necessary, for additional information. The primary endpoint was the occurrence of MACE, defined as the composite of all-cause mortality, nonfatal ACS or unplanned coronary revascularization. The secondary endpoint was the composite of all-cause mortality or nonfatal ACS.

Endpoints were adjudicated by a clinical events committee that was blinded for biomarker data and IVUS/NIRS imaging characteristics. ACS was defined as the clinical diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris (UAP) in accordance with the guidelines of the European society of Cardiology[16, 17]. Unplanned coronary revascularization was defined as unplanned PCI or unplanned coronary artery bypass grafting (CABG).

Statistical analysis

The distributions of continuous variables, including biomarkers and NIRS measurements, 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 (interquartile range [IQR]) and were logarithmically (Ln) transformed for further analyses. For reasons of uniformity, all biomarkers are presented as median (IQR). Categorical variables are presented as numbers and percentages. All analyses were performed in the full cohort and subsequently in patients with ACS and patients with SAP separately, to investigate possible heterogeneity.

We examined the association between biomarker concentrations and LCBI as assessed by NIRS using linear regression with LCBI as the independent variable and continuous biomarkers as the dependent variable. The concentrations of

CRP, A2Macro, ferritin,haptoglobin,PAI-1, MIP-1α, MIP-1β, MCP-1, T-Cell-Specific RANTES, TNF-α, TNF-β, TNFR2, INF-γ, IL-6, IL-8, IL-10, IL-18, fibrinogen, creatinine, Cystatin C, NGAL, Adiponectin, myoglobin, and B2M were not normally distributed and therefore Ln transformed. TNF-β and IL-6 were too low to detect in a large part of the patients and thus were not examined as continuous variable but as categorical variables (measurable vs non-measurable).The results are presented as beta coefficients (B) that indicate unit increase in (Ln-transformed) biomarker per unit increase in Ln-transformed LCBI measurement, with 95 % confidence intervals (CI).

Cox proportional hazards models were used to examine the associations between biomarker concentrations and MACE, as well as the composite of all-cause mortality or nonfatal ACS. Results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) biomarker concentration or per category of biomarker concentration, with 95 % CIs. Patients lost to follow-up were considered at risk until the date of last contact, at which time point they were censored. For patients with more than 1 event, the first was considered. To test effect modification, interaction terms were entered into the models consisting of the product of biomarker and indication for angiography (ACS or SAP).

First, all above-described analyses were performed univariably. Based on existing literature, age (continuous variable), as well as sex, hypertension, hypercholesterolemia and diabetes mellitus (all categorical variables) were considered as potential confounders and were subsequently entered as covariates into the multivariable analyses. In the full cohort, indication for coronary angiography was also entered as a covariate.

All statistical tests were two-tailed. P-values <0.05 were considered statistically significant and the results are presented with 95% confidence intervals (95%CIs). Subsequently, the Bonferroni correction was applied to account for the 26 biomarkers that were investigated (and thus p-values <0.05/26, i.e. P<0.0019 were considered statistically significant). Data were analyzed with SPSS software (SPSS 23.0 IBM corp., Armonk, NY, USA).

RESULTS

Baseline characteristics

Baseline clinical and imaging characteristics are summarized in Table-1 and Supplemental Table-1. In ATHEROREMO-NIRS (n=203), mean age was 63.4 years, and 72.9% were men. The biomarker concentrations are presented in Supplemental

Table-2 and Supplemental Table-3. In the full cohort (n=570) serum concentrations of MIP-1α, TNF-α, TNF-β, IL-6 and NGAL were measurable in 84%, 92%, 8%, 38% and 97% of the patients, respectively. The remaining biomarker concentrations were measurable in ≥99% of the patients. In the ATHEROREMO-NIRS cohort, concentrations of TNF-β, IL-6 and NGAL were measurable in 6%, 32% and 96% of the patients, respectively. The remaining biomarker concentrations were measurable in ≥99% of the patients.

Association between coronary LCBI and biomarkers

The results of the multivariable linear regression analyses are depicted in Figure-3 and Table-2. Higher TNF-α (multivariable adjusted B: 0.088 ln(pg/ml) per unit LCBI; 95% CI: 0.000-0.177, p=0.05) displayed a tendency towards an association at the p=0.05 level with higher LCBI in the full cohort after adjustment for clinical characteristics. Effect estimates did not reach statistical significance at the p=0.05 level in ACS and SAP patients (Figure-3 and Table-2). After Bonferroni correction, no

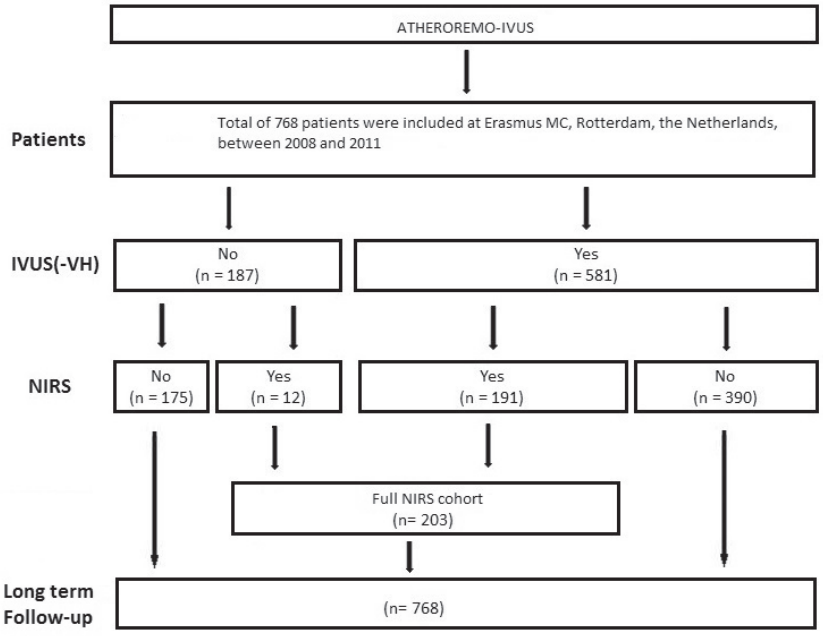


Figure 1. Patient inclusion
IVUS of a non-culprit artery was performed in 581 patients and blood samples were available in 570 patients. NIRS of a non-culprit artery was performed in 203 patients and blood samples were available in 190 patients

associations were present between any of the biomarkers and LCBI. Results of the univariable analyses were materially the same (results not presented).

Biomarkers and major adverse cardiac events

Vital status was acquired for 569 out of 570 patients (99.8 %). The follow-up questionnaire assessing the occurrence of MACE was completed by 87.5% of the 570 patients. During a median follow-up time of 4.7 years IQR: [4.2-5.6] years, 155 patients (27%) experienced at least 1 MACE (primary endpoint). Hazard ratios for the occurrence of MACE are shown in Figure-4 and Supplemental Tables-4a and 4b. After Bonferroni correction, only IL-8 (p=0.002) was borderline significantly associated with MACE. No independent associations were present between the other biomarkers and MACE. At the p=0.05 level, some biomarkers tended to display associations with MACE. Specifically, on univariable analysis, higher T-Cell Specific Rantes, IFN-ɣ, IL-8, Cystatin C and B2M were associated with higher incidence of MACE at

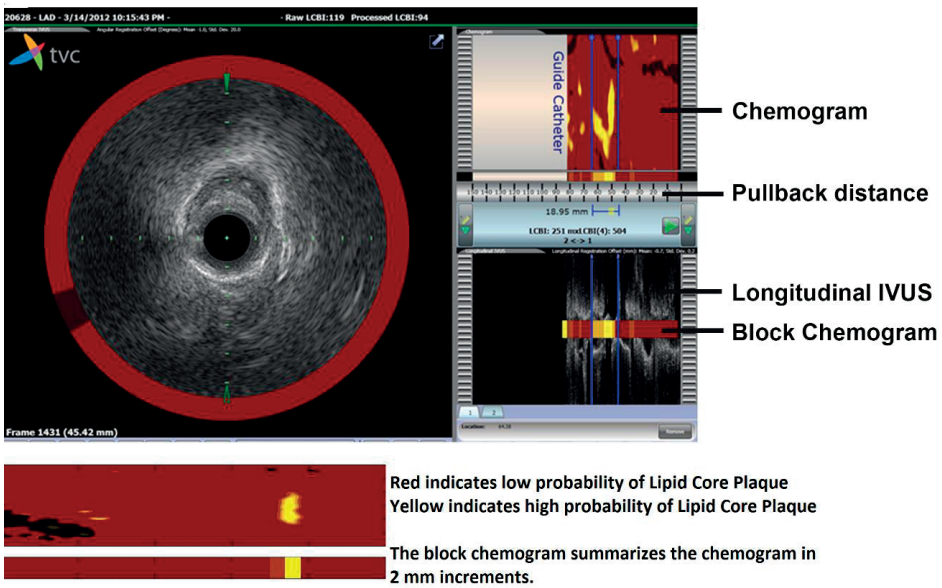


Figure 2. Intracoronary near-infrared spectroscopy displayed as a chemogram
The figure displays an example of coronary wall imaging by near-infrared spectroscopy. Spectral characteristics of lipid core plaques are displayed on a chemogram along the length (x-axis, in mm) and circumference (y-axis, 0 to 360 degrees) of the scanned coronary artery. Yellow regions in the chemogram represent high probability of LCP while red regions represent those with low probability of LCP. The LCBI quantifies the amount of LCP in the entire scanned artery segment on the block chemogram, and is computed as the fraction of valid pixels that exceed an LCP probability of 0.6, multiplied with 1000

Table 1. Baseline clinical and procedural characteristics (ATHEROREMO-NIRS cohort, n=203)

	Total (n=203)	ACS patients (n= 95)	SAP patients (n= 108)
Clinical characteristics			
Age, years, mean ± standard deviation	63.4 ± 10.9	62 ± 11.7	64.7 ± 10.2
Male	148(72.9)	63(66.3)	85(78.7)
Diabetes Mellitus	41(20.2)	17(17.9)	24(22.2)
Hypertension	114(56.2)	51(53.7)	63(58.3)
Hypercholesterolemia	115(56.7)	43(45.3)	72(66.7)
Smoking	50(24.6)	30(31.6)	20(18.5)
Positive family history of CAD	120(59.1)	51(54.3)	69(63.9)
Previous MI	79(38.9)	34(35.8)	45(41.7)
Previous PCI	78(38.4)	27(28.4)	51(47.2)
Previous CABG	6(3.0)	2(2.1)	4(3.7)
Previous stroke	6(3.0)	4(4.2)	2(1.9)
Peripheral artery disease	11(5.4)	5(5.3)	6(5.6)
History of heart failure	9(5.9)	3(3.2)	6(5.6)
Procedural characteristics			
Indication for coronary angiography			
ACS	95(46.8)	95(100)	-
Acute MI	28(13.8)	28(29.5)	-
Unstable angina pectoris	67(33.0)	67(70.5)	-
Stable angina pectoris	108(53.2)	-	108(100)
PCI performed	179(88.2)	88(92.6)	91(84.3)
Coronary artery disease[1]			
No significant stenosis	16(7.9)	8(8.4)	8(7.4)
1-vessel disease	106(52.2)	49(51.6)	57(52.8)
2-vessel disease	58(28.6)	26(27.4)	32(29.6)
3-vessel disease	23(11.3)	12(12.6)	11(10.2)
NIRS characteristics			
Median LCBI [IQR]	43.0[15.0-90.0]	47.0[16.0-90.0]	35.0[14.0-85.5]
Imaged coronary artery			
Left anterior descending	74(36.5)	41(43.2)	33(30.6)
Left circumflex	70(34.5)	30(31.6)	40(37.0)
Right coronary artery	59(29.1)	24(25.3)	35(32.4)

Continuous variables are presented as mean± standard deviation (SD) or median [IQR]. Categorical variables are presented in numbers (n) and percentages (%).

ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; IQR, interquartile range; LCBI, Lipid Core Burden Index; MI, myocardial infarction; PCI, percutaneous coronary intervention; SAP, stable angina pectoris.

1 A significant stenosis was defined as a stenosis ≥ 50% of the vessel diameter by visual assessment of the coronary angiogram.

Figure 3a. Association of 26 biomarkers with LCBI in the full NIRS cohort

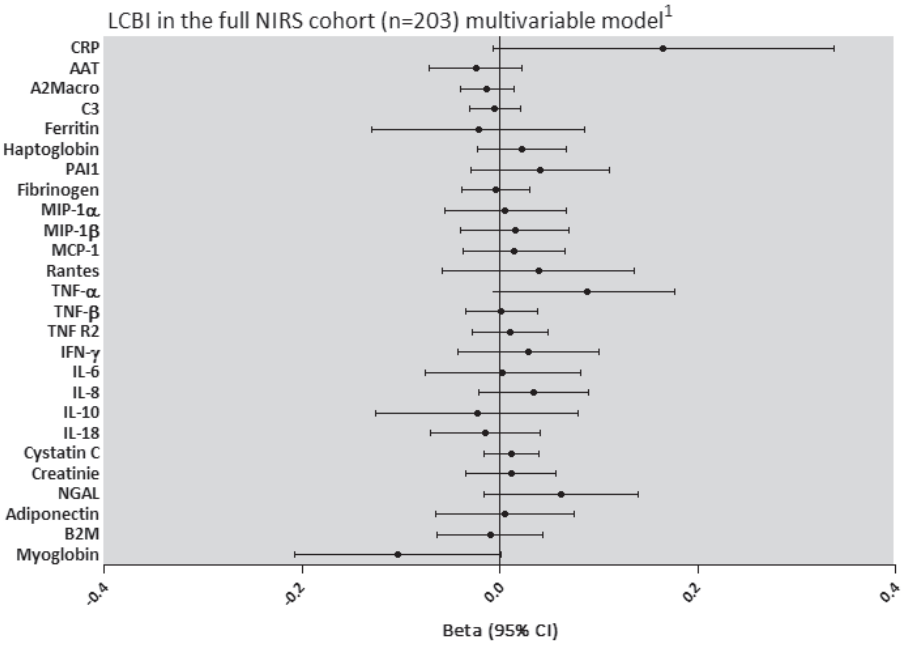


Figure 3b. Association of 26 biomarkers with LCBI in the ACS patients

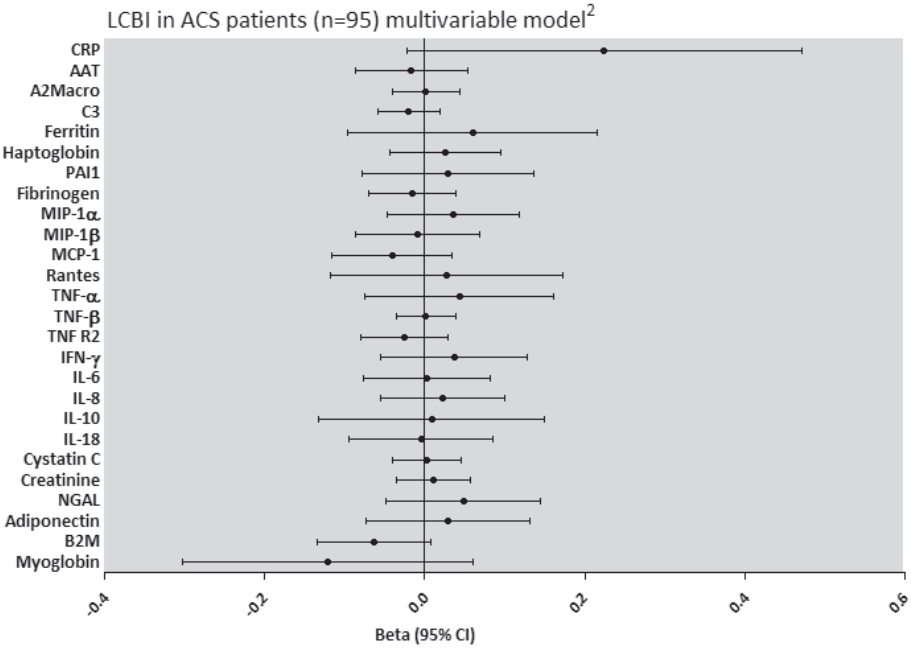
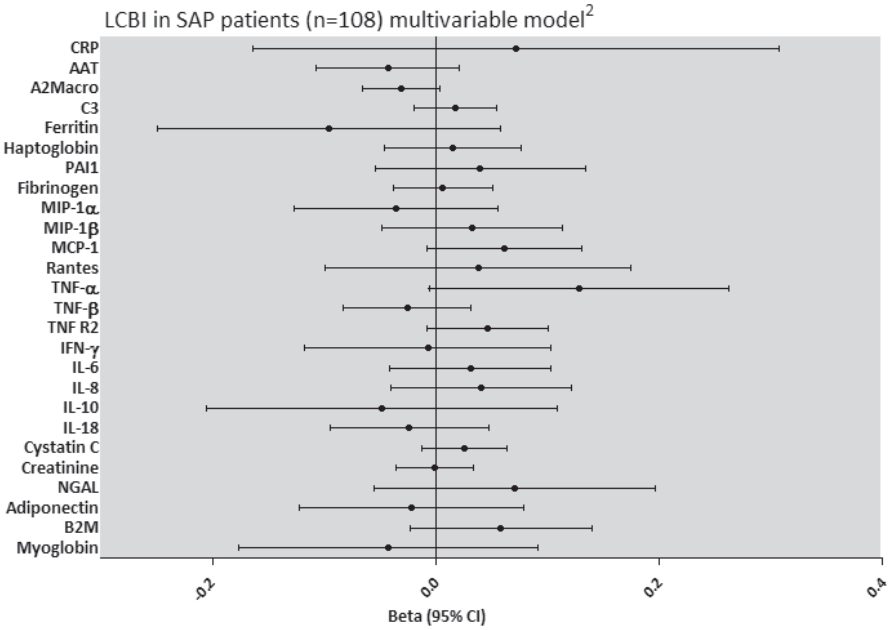


Figure 3c. Association of 26 biomarkers with LCBI in SAP patients



[1] Model is adjusted for age, gender, diabetes mellitus, hypertension, hypercholesterolemia and indication for coronary angiography.
[2] Models are adjusted for age, gender, diabetes mellitus, hypertension and hypercholesterolemia.

TNF-β was measurable in 6%, and IL 6 in 32% of the patients; thus there biomarkers were not examined as continuous variables but as categorical variables (measurable vs not measurable).

AAT, Alpha- 1- Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha- 2- Macroglobulin; B2M, beta-2-Microglobulin; CRP, C –reactive protein; IFN-γ, Interferon γ; IL, interleukin; IQR, interquartile range; LCBI, lipid core burden index; MACE, major adverse cardiac events; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF α, Tumor Necrosis Factor alpha; TNF β, Tumor Necrosis Factor beta; TNFR 2,Tumor Necrosis Factor Receptor2.

Figure 3. Association of 26 biomarkers with LCBI in the full NIRS cohort and in patients with ACS or SAP

Results are presented as Beta, per unit increase in (Ln-transformed) biomarker concentration or per category of biomarker concentration per unit increase in Ln-transformed LCBI measurement, with 95% confidence intervals (CI)

Table 2. Association between biomarkers and LCBI (NIRS cohort n=203)

	Total (n=203)		ACS patients (n= 95)		SAP patients (n= 108)	
	B [95% CI][2]	P-value	B [95% CI][3]	P-value	B [95% CI][2]	P-value
Acute phase proteins						
CRP [4]	0.165 (-0.007-0.337)	0.06	0.224 (-0.023-0.471)	0.08	0.071 (-0.164-0.307)	0.55
AAT[5]	-0.025 (-0.071-0.022)	0.30	-0.017 (-0.087-0.054)	0.64	-0.043 (-0.107-0.021)	0.18
A2Macro[4]	-0.013 (-0.040-0.014)	0.35	0.001 (-0.040-0.043)	0.95	-0.031 (-0.065-0.004)	0.08
Complement C3[4]	-0.005 (-0.031-0.021)	0.72	-0.020 (-0.059-0.019)	0.32	0.018 (-0.019-0.054)	0.34
Ferritin[6]	-0.022 (-0.129-0.086)	0.69	0.060 (-0.096-0.215)	0.45	-0.096 (-0.249-0.058)	0.22
Haptoglobin[5]	0.022 (-0.023-0.067)	0.34	0.025 (-0.044-0.095)	0.47	0.015 (-0.046-0.076)	0.62
PAI 1[5]	0.041 (-0.029-0.111)	0.25	0.029 (-0.078-0.136)	0.59	0.040 (-0.054-0.134)	0.40
Fibrinogen[5]	-0.005 (-0.039-0.030)	0.79	-0.015 (-0.070-0.039)	0.58	0.006 (-0.038-0.051)	0.78
Chemokine						
MIP-1 α[5]	0.006 (-0.056-0.067)	0.86	0.036 (-0.047-0.118)	0.39	-0.035 (-0.127-0.056)	0.44
MIP-1 β[5]	0.015 (-0.040-0.070)	0.60	-0.008 (-0.086-0.069)	0.83	0.033 (-0.048-0.113)	0.42
MCP-1[5]	0.015 (-0.037-0.066)	0.57	-0.041 (-0.116-0.034)	0.28	0.062 (-0.008-0.131)	0.08
T-Cell-Specific RANTES[5]	0.039 (-0.058-0.136)	0.43	0.028 (-0.118-0.173)	0.71	0.038 (-0.099-0.175)	0.59
Cytokines						
TNF-α[4]	0.088 (0.000-0.177)	0.05	0.043 (-0.075-0.161)	0.47	0.128 (-0.005-0.262)	0.06
TNF-β[7]	-0.007 (-0.039-0.026)	0.68	0.001 (-0.035-0.038)	0.94	-0.026 (-0.083-0.032)	0.37
TNF R2[5]	0.011 (-0.028-0.049)	0.59	-0.025 (-0.080-0.029)	0.36	0.047 (-0.008-0.101)	0.09
IFN-ɣ[4]	0.029 (-0.042-0.100)	0.42	0.037 (-0.055-0.128)	0.42	-0.007 (-0.117-0.103)	0.90
IL-6[6]	0.018 (-0.034-0.071)	0.49	0.003 (-0.076-0.082)	0.94	0.031 (-0.041-0.103)	0.39
IL-8[5]	0.034 (-0.021-0.090)	0.23	0.022 (-0.056-0.100)	0.57	0.041 (-0.040-0.121)	0.32
IL-10[4]	-0.023 (-0.125-0.079)	0.65	0.009 (-0.132-0.149)	0.90	-0.048 (-0.205-0.109)	0.55
IL-18[4]	-0.015 (-0.070-0.041)	0.61	-0.005 (-0.094-0.085)	0.92	-0.023 (-0.095-0.048)	0.52
Renal markers						
Creatinine[8]	0.004 (-0.024-0.032)	0.77	0.011 (-0.035-0.057)	0.64	-0.001 (-0.035-0.034)	0.96
Cystatin C[5]	0.011 (-0.016-0.039)	0.42	0.002 (-0.040-0.045)	0.91	0.026 (-0.012-0.064)	0.17
NGAL[4]	0.062 (-0.016-0.139)	0.12	0.048 (-0.049-0.145)	0.33	0.070 (-0.055-0.196)	0.27
Other markers						
Adiponectin[5]	0.005 (-0.065-0.075)	0.87	0.028 (-0.074-0.131)	0.59	-0.021 (-0.122-0.079)	0.67
Myoglobin[5]	-0.096 (-0.207-0.015)	0.09	-0.120 (-0.302-0.061)	0.19	-0.042 (-0.176-0.091)	0.53
B2M[4]	-0.010 (-0.063-0.043)	0.72	-0.064 (-0.135-0.007)	0.08	0.059 (-0.023-0.140)	0.15

Variables with a non-normal distribution were transformed by the natural logarithm (ln). Results are presented as beta coefficients (B) that indicate unit increase in (ln-transformed) biomarker level or per category of biomarker concentration per unit Ln-transformed LCBI, with 95% confidence intervals (CI). AAT, Alpha- 1- Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha-2- Macroglobulin; B2M, beta-2-Microglobulin; CRP, C –reactive protein; IFN-ɣ, Interferon ɣ; IL, interleukin; IQR, interquartile range; LCBI, lipid core burden index; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF-α, Tumor Necrosis Factor alpha; TNF-β, Tumor Necrosis Factor beta; TNF R2,Tumor Necrosis Factor Receptor2.1 2 3 4 5 6 7

1 Model is adjusted for age, gender, diabetes mellitus, hypertension, hypercholesterolemia and indication for coronary angiography.
2 Models are adjusted for age, gender, diabetes mellitus, hypertension and hypercholesterolemia.
3 Available in the full cohort.
4 Available in 156 patients.
5 Available in 190 patients.
6 Too low to detect in a large part of the patients (TNF-β was measurable in 6% and IL 6 in 32% of the patients) and thus not examined as a continuous variable but as a categorical variable (measurable vs not measurable).
7 Available in 188 patients.

Figure 4a. Association of 26 biomarkers with MACE in the full cohort

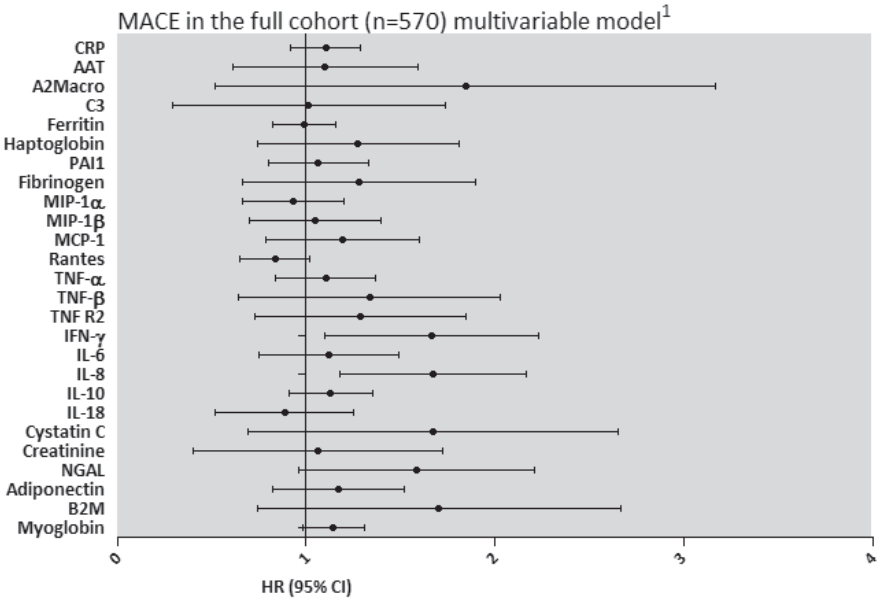
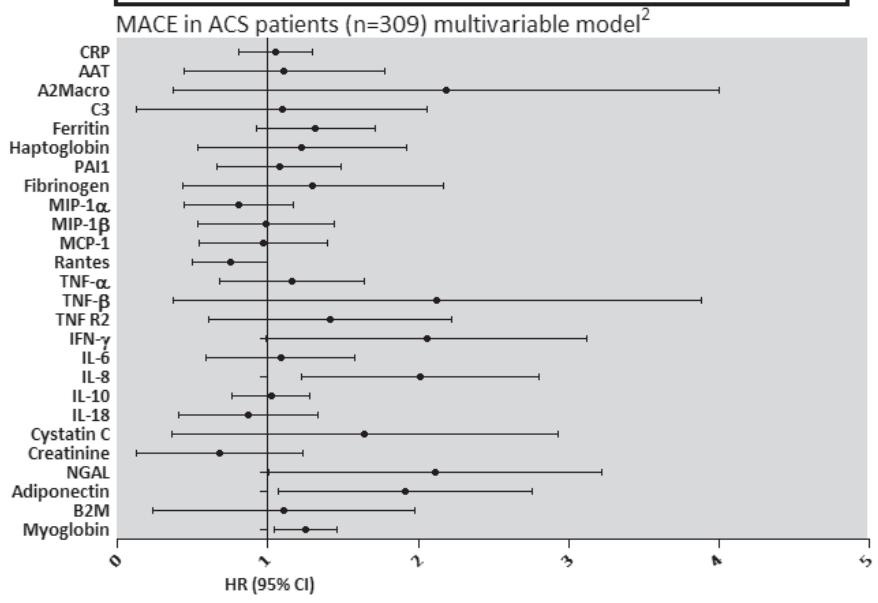
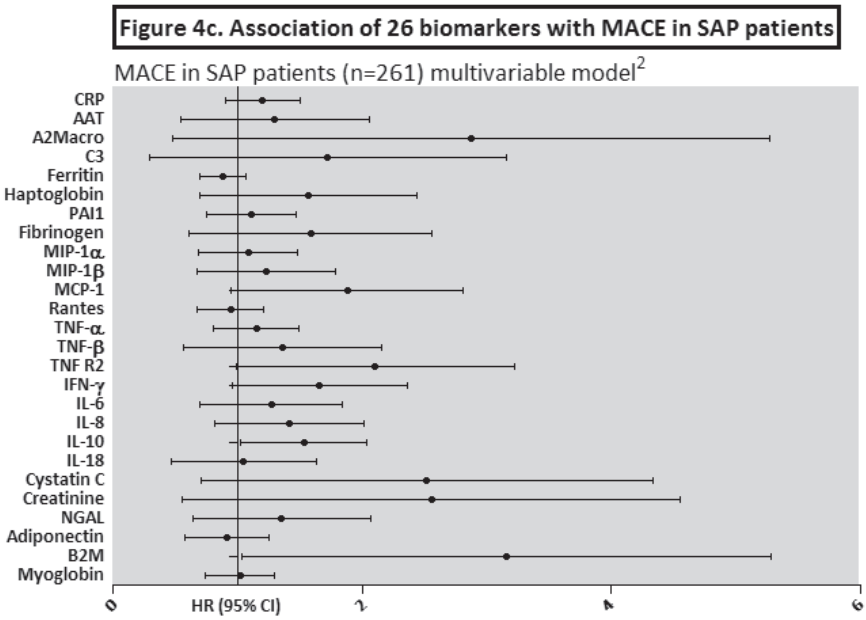


Figure 4b. Association of 26 biomarkers with MACE in ACS patients





[1] Model is adjusted for age, gender, diabetes mellitus, hypertension, hypercholesterolemia and indication for coronary angiography.
[2] Models are adjusted for age, gender, diabetes mellitus, hypertension and hypercholesterolemia.

TNF-β was measurable in 8%, and IL 6 in 38% of the patients; thus these biomarkers were not examined as continuous variables but as categorical variables (measurable vs not measurable).

AAT, Alpha- 1- Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha- 2- Macroglobulin; B2M, beta-2-Microglobulin; CRP, C –reactive protein; IFN-γ, Interferon γ; IL, interleukin; IQR, interquartile range; MACE, major adverse cardiac events; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF α, Tumor Necrosis Factor alpha; TNF β, Tumor Necrosis Factor beta; TNFR 2,Tumor Necrosis Factor Receptor2.

Figure 4. Association of 26 biomarkers with MACE in the full cohort and in patients with ACS or SAP

Results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) biomarker concentration or per category of biomarker concentration, with 95% confidence intervals (CI)

the p=0.05 level. After adjustment for clinical characteristics, IFN-ɣ (HR: 1.57; 95%CI [1.10-2.23 per ln(pg/ml)) and IL-8 (HR: 1.60; 95%CI [1.18-2.17 per ln(pg/ml)) remained independently associated with MACE at the p=0.05 level. Interaction terms between biomarkers and indication for angiography only reached significance for IL-10 (p for interaction =0,05). In patients diagnosed with ACS or SAP, there were no significant association between any of the biomarkers and MACE

Only IL-8 (p=0.0015) was significantly associated with the composite of death or ACS (secondary endpoint) after Bonferroni correction and adjustment for clinical characteristics (Supplemental Table-5). At the p=0.05 level, higher CRP, A2Macro, fibrinogen, TNFR2, INF-γ, IL-8, Cystatin C, adiponectin and B2M were associated with this secondary endpoint on univariable analysis (data not shown). Interaction terms between biomarkers and indication for angiography reached significance for B2M (p for interaction =0.036). In patients with ACS, only IL-8 (HR: 2.89; 95%CI [1.86-4.14 per ln (pg/ml) p≤0.001) remained associated with the composite of death or ACS after multivariable adjustment and Bonferroni correction. In patients with SAP, the HR for IL-8 was closer to the null (HR: 1.12; 95%CI [0.61-2.03 per ln (pg/ml) p=0.72). Effect estimates for CRP and INF-γ were similar to those in the full cohort in ACS and SAP patients, but statistical significance was not reached. For B2M, the multivariable adjusted hazard ratio was significantly higher in SAP patients than in ACS patients, but did not reach statistical significance after Bonferroni correction.

DISCUSSION

We investigated the association of 26 circulating biomarkers with NIRS-derived LCBI in 203 patients undergoing coronary angiography. After multivariable adjustment and correction for multiple testing, none of the 26 biomarkers was associated with LCBI. Furthermore, we also investigated the long-term prognostic value of these 26 biomarkers for clinical cardiovascular outcome in 570 patients. After correction for multiple testing, we found that IL-8 was borderline significantly associated with MACE, and independently associated with death or ACS.

Studies on the association between circulating biomarkers and NIRS-derived characteristics have not been performed previously. NIRS has recently progressed from bench testing to human studies. In 1993 Cassis and Lodder [18] first described the use of NIRS for characterisation of atherosclerotic plaque in rabbit aortas. Ever since, there have been several [4, 15] studies that have validated the use of NIRS for identification of lipid deposition within the coronary arteries. NIRS has been shown

to identify extensive LCPs that are associated with a high risk of peri-procedural myocardial infarction [15]. In our previous report on the current study population, NIRS-derived LCBI was an independent predictor of MACE during 1 year follow-up [6]. As NIRS has the potential to identify LCPs indicative of plaque vulnerability in the coronary arteries, we hypothesized that circulating inflammatory biomarkers are associated with NIRS-derived LCBI. However, we could not demonstrate any associations between these biomarkers and NIRS-derived LCBI after Bonferroni correction. These results suggest that any potential effects of these biomarkers on atherosclerosis are exerted through other mechanisms.

TNF-α is a pro-inflammatory cytokine with pleiotropic actions. In a previous report on the current patient population, TNF-α concentration was positively associated with plaque burden and plaque vulnerability as determined by IVUS [8]. In the current study we found that at the p=0.05 level, TNF-α displayed a tendency towards a positive association with LCBI after multivariable adjustment, but this association did not persist after correction for multiple testing. Previously, Sukhija et al. [19] found no association between serum TNF-α levels and extent of atherosclerosis or clinical outcome in patients with known coronary artery disease (CAD). Conversely, other studies [20, 21] have demonstrated positive associations between plasma concentration of TNF-α and coronary events. However, in our study TNF-α was not associated with MACE at long-term follow-up, implying that the deleterious effect of TNF-α, if any, does not translate into a higher MACE rate in the current study population. More research is necessary to further substantiate the pathological mechanisms underlying the roles of this biomarker in atherosclerotic plaque development. No associations could be demonstrated between any of the other biomarkers and NIRS-derived LCBI.

In our previous reports on the ATHEROREMO-IVUS study [11, 8-10, 12], levels of CRP, ferritin, Rantes, TNF-α, IL-10, Cystatin C and NGAL were associated with IVUS-VH derived plaque burden in the full cohort. Additionally, IL-10, Cystatin C and NGAL were associated with IVUS-VH derived thin-cap fibroatheroma (VH-TCFA) lesions. This difference in findings could in part be explained by the differences in definitions used for NIRS- and IVUS-derived measures of plaque vulnerability. NIRS-derived LCBI represents the amount of LCP in the entire scanned artery segment, and is computed as the fraction of valid pixels on the block chemogram that exceed an LCP probability of 0.6, multiplied with 1000. IVUS-VH-derived TCFA lesions are defined as lesions with presence of >10% confluent necrotic core in direct contact with the lumen [9]. Although these entities are related, previous studies have

shown that the correlation between LCP as detected by NIRS and necrotic core as detected by IVUS-VH is weak [14].

We found that IL-8 is associated with clinical outcome during long-term follow-up. Inoue T. et al. [22] investigated the long-term prognostic value of IL-8 in patients with CAD and found IL-8 as only cytokine predictor of cardiovascular events, independently of other cytokines and hs-CRP. Cavusoglu et al. [23] found an association between high baseline plasma levels of IL-8 with increased risk of long-term all-cause mortality in patients with ACS. Our findings are in line with these results.

Study limitations

This study has several limitations. First, this is a cross sectional study. As we did not repeat NIRS imaging of the same segment at a later time point, no information is available on the change in LCBI and its relation with biomarkers levels over time. Future research might focus on the effects of changes in biomarker level and their effect on LCBI. Secondly, the NIRS image acquisition was performed in only one non-culprit vessel. This study design was chosen based on the hypothesis that such a non-stenotic segment reflects coronary wall pathophysiology in the larger coronary tree [13]. This assumption, on its part, was based on the fact that ex-vivo, as well as in-vivo studies in patients with myocardial infarction, have demonstrated presence of TCFA (as assessed by IVUS) located elsewhere than the culprit lesion or even culprit artery [9, 8, 10-12]. In fact, we were subsequently able to confirm this hypothesis, by demonstrating that NIRS imaging characteristics of the non-culprit artery are associated with increased risk of MACE [6].

Conclusion

None of the 26 biomarkers we examined was associated with LCBI after correction for multiple testing. IL-8 was associated with clinical outcome in patients undergoing coronary angiography after correction for multiple testing. Altogether, the multiplex panel we investigated here did not render a useful blood biomarker of high LCBI.

REFERENCES

1. Hellings WE, Peeters W, Moll FL, Pasterkamp G. From vulnerable plaque to vulnerable patient: the search for biomarkers of plaque destabilization. Trends Cardiovasc Med. 2007;17(5):162-71.
2. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. Circulation. 2003;108(15):1772-8.
3. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol. 2000;20(5):1262-75.
4. Moreno PR, Lodder RA, Purushothaman KR, Charash WE, O'Connor WN, Muller JE. Detection of lipid pool, thin fibrous cap, and inflammatory cells in human aortic atherosclerotic plaques by near-infrared spectroscopy. Circulation. 2002;105(8):923-7.
5. Celeng C, Takx RA, Ferencik M, Maurovich-Horvat P. Non-invasive and invasive imaging of vulnerable coronary plaque. Trends Cardiovasc Med. 2016;26(6):538-47.
6. Oemrawsingh RM, Cheng JM, Garcia-Garcia HM, van Geuns RJ, de Boer SP, Simsek C et al. Near-infrared spectroscopy predicts cardiovascular outcome in patients with coronary artery disease. J Am Coll Cardiol. 2014;64(23):2510-8.
7. Simsek C, Garcia-Garcia HM, Brugaletta S, de Boer SP, Magro M, Duckers HJ et al. Correlation between kidney function and near-infrared spectroscopy derived lipid-core burden index score of a non-intervened coronary artery segment. Int J Cardiol. 2012;156(2):226-8.
8. Battes LC, Cheng JM, Oemrawsingh RM, Boersma E, Garcia-Garcia HM, de Boer SP et al. Circulating cytokines in relation to the extent and composition of coronary atherosclerosis: results from the ATHEROREMO-IVUS study. Atherosclerosis. 2014;236(1):18-24.
9. Battes LC, Akkerhuis KM, Cheng JM, Garcia-Garcia HM, Oemrawsingh RM, de Boer SP 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(3):847-53.
10. Cheng JM, Oemrawsingh RM, Akkerhuis KM, Garcia-Garcia HM, de Boer SP, Battes LC et al. Circulating chemokines in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and cardiovascular outcome. Biomarkers. 2014;19(7):611-9.
11. Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Akkerhuis KM, Kardys I, de Boer SP 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(10):1497-503.
12. Brankovic M, Akkerhuis KM, Buljubasic N, Cheng JM, Oemrawsingh RM, Garcia-Garcia HM et al. 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;254:20-7.

13. de Boer SP, Cheng JM, Garcia-Garcia HM, Oemrawsingh RM, van Geuns RJ, Regar E 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(8):953-60.
14. Brugaletta S, Garcia-Garcia HM, Serruys PW, de Boer S, Ligthart J, Gomez-Lara J et al. NIRS and IVUS for characterization of atherosclerosis in patients undergoing coronary angiography. JACC Cardiovasc Imaging. 2011;4(6):647-55.
15. Goldstein JA, Maini B, Dixon SR, Brilakis ES, Grines CL, Rizik DG et al. Detection of lipid-core plaques by intracoronary near-infrared spectroscopy identifies high risk of periprocedural myocardial infarction. Circ Cardiovasc Interv. 2011;4(5):429-37.
16. Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). Eur Heart J. 2016;37(3):267-315.
17. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J. 2017.
18. Cassis LA, Lodder RA. Near-IR imaging of atheromas in living arterial tissue. Anal Chem. 1993;65(9):1247-56.
19. Sukhija R, Fahdi I, Garza L, Fink L, Scott M, Aude W et al. Inflammatory markers, angiographic severity of coronary artery disease, and patient outcome. The American journal of cardiology. 2007;99(7):879-84. doi:10.1016/j.amjcard.2006.11.032.
20. Mizia-Stec K, Gasior Z, Zahorska-Markiewicz B, Janowska J, Szulc A, Jastrzebska-Maj E 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(6):431-8. doi:10.1097/01.mca.0000085707.34267.70.
21. Cesari M, Penninx BW, Newman AB, Kritchevsky SB, Nicklas BJ, Sutton-Tyrrell K et al. Inflammatory markers and onset of cardiovascular events: results from the Health ABC study. Circulation. 2003;108(19):2317-22.
22. Inoue T, Komoda H, Nonaka M, Kameda M, Uchida T, Node K. Interleukin-8 as an independent predictor of long-term clinical outcome in patients with coronary artery disease. Int J Cardiol. 2008;124(3):319-25.
23. Cavusoglu E, Marmur JD, Yanamadala S, Chopra V, Hegde S, Nazli A et al. Elevated baseline plasma IL-8 levels are an independent predictor of long-term all-cause mortality in patients with acute coronary syndrome. Atherosclerosis. 2015;242(2):589-94. doi:10.1016/j.atherosclerosis.2015.08.022.

SUPPLEMENTAL FILE

Supplemental Table 1. Baseline clinical and procedural characteristics (full cohort, n=570)

	Total (n=570)	ACS patients (n=309)	SAP patients (n= 261)
Clinical characteristics			
Age, years, mean ± standard deviation	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Male, n (%)	430 (75.4%)	227 (73.5)	203 (77.8)
Diabetes Mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Hypercholesterolemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Positive family history of CAD, n (%)	293 (51.5)	140 (45.5)	153 (58.6)
Previous MI, 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)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
Procedural characteristics			
Indication for coronary angiography			
ACS, n (%)	309 (54.2)	309 (100)	0 (0)
Acute MI, 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)
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
Coronary artery disease[1]			
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)
NIRS characteristics			
Median LCBI (IQR)	43.0 (15.0-84.0)	47.5 (16.0-90.5)	35.0 (14.0-80.8)
Imaged coronary artery			
Left anterior descending, n (%)	204 (35.9)	117 (37.9)	87 (33.6)
Left circumflex, n (%)	190 (33.5)	107 (34.6)	83 (32.0)
Right coronary artery, n (%)	174 (30.6)	85 (27.5)	89 (34.4)

Continuous variables are presented as mean ± standard deviation (SD) or median [IQR]. Categorical variables are presented in numbers (n) and percentages (%).

ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; IQR, interquartile range; LCBI, Lipid Core Burden Index; MI, myocardial infarction; PCI, percutaneous coronary intervention; SAP, stable angina pectoris.

1 A significant stenosis was defined as a stenosis ≥ 50% of the vessel diameter by visual assessment of the coronary angiogram.

Supplemental Table 2. Biomarker concentrations (ATHEROREMO-NIRS cohort, n=203)

	Total (n=203)	ACS patients (n=95)	SAP patients (n=108)
Acute phase proteins			
CRP (mg/l)[1]	2.10[0.80-5.35]	3.00[1.00-6.65]	1.60[0.60-4.00]
AAT (mg/ml)[2]	1.50[1.20-1.80]	1.60[1.20-1.90]	1.40[1.20-1.65]
A2Macro (mg/ml)[2]	1.60[1.40-1.80]	1.60[1.40-1.80]	1.60[1.50-1.80]
Complement C3 (mg/ml)[2]	0.94[0.78-1.10]	0.90[0.78-1.10]	0.96[0.80-1.10]
Ferritin (mg/ml)[3]	178.50[94.0-280.75]	191.5[98.75-356.0]	150.50[83.50-237.75]
Haptoglobin (mg/ml)[3]	1.43[0.87-2.19]	1.60[0.96-2.50]	1.30[0.87-2.10]
PAI 1 (ng/ml)[3]	31.0[22.0-45.25]	31.0[23.0-44.15]	30.50[20.40-46.75]
Fibrinogen (pg/ml)[3]	3.90[3.00-4.70]	4.05[3.0-5.11]	3.76[2.93-4.41]
Chemokine			
MIP-1α (pg/ml)[3]	16.0[12.0-23.0]	15.0[11.0-21.90]	17.50[12.0-24.50]
MIP-1β (pg/ml)[3]	119.50[92.0-154.25]	126.0[96.0-165.50]	112.50[90.18-142.0]
MCP-1 (pg/ml)[3]	86.5[67.0-108.0]	85.0[63.75-106.25]	87.50 [71.25-113.25]
T-Cell-Specific RANTES (ng/ml)[3]	9.43[4.98-15.0]	10.50[5.83-17.25]	9.34[4.70-14.0]
Cytokines			
TNF-α (pg/ml)[2]	2.00[1.40-2.80]	1.80[1.40-2.60]	2.0[1.40-3.30]
TNF-β (pg/ml)[4]	36.0[18.0-119.0]	37.0[18.0-…]	35.0[18.0-128.0]
TNF R2 (ng/ml)[6]	4.40[3.60-5.60]	4.40[3.60-6.00]	4.35[3.53-5.52]
IFN-ɣ (pg/ml)[2]	5.40[3.90-7.90]	5.10[3.90-7.30]	5.60[3.90-8.50]
IL-6 (pg/ml)[5]	3.10[2.13-5.45]	9.40[7.85-13.0]	2.20[2.13-3.84]
IL-8 (pg/ml)[3]	9.00[6.90-12.0]	3.57[2.43-6.90]	8.34[6.15-10.83]
IL-10 (pg/ml)[2]	5.00[3.60-7.25]	5.70[3.70-8.0]	4.80[3.20-6.80]
IL-18 (pg/ml)[2]	155.0[122.25-190.50]	150.0[108.0-191.0]	156.0[131.0-189.0]
Renal markers			
Cystatin C (ng/ml)[3]	792.50[687.75-926.5]	788.5[665.5-931.5]	806.0[711.25-921.75]
Creatinine (umol/l)[6]	75.00[62.00-84.50]	72.50[61.00-84.50]	76.00[62.50-84.50]
NGAL (pg/ml)[7]	194.50[137.0-239.0]	201.0[143.0-254.0]	175.0[125.0-238.5]
Other markers			
Adiponectin (pg/ml)[3]	2.90[1.80-4.10]	2.75[1.80-4.03]	3.00[1.90-4.18]
B2M (pg/ml)[2]	1.35[1.10-1.70]	1.30[1.0-1.70]	1.40[1.20-1.80]
Myoglobin (pg/ml)[3]	32.50[19.75-60.50]	33.50[21.0- 69.25]	31.0[18.0-55.58]

All biomarkers are presented as median [IQR].

AAT, Alpha- 1- Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha- 2- Macroglobulin; B2M, beta-2-Microglobulin; CRP, C –reactive protein; IFN-ɣ, Interferon ɣ; IL, interleukin; IQR, interquartile range; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF-α, Tumor Necrosis Factor alpha; TNF-β, Tumor Necrosis Factor beta; TNF R2,Tumor Necrosis Factor Receptor2.

1 Available in 201 patients
2 Available in a random subset of 156 patients
3 Available in 190 patients
4 TNF-β was measurable in 6% of 156 patients, too low to detect in 94%.
5 IL-6 was measurable in 32% of 190 patients, too low to detect in 68%.
6 Available in 99 % of 190 patients, missing in 1 %.
7 NGAL was measurable in 96% of 156 patients, too low to detect in 4%.

Supplemental Table 3. Biomarker concentrations in the full cohort (n=570)

	Total (n=570)	ACS patients (n=309)	SAP patients (n= 261)
Acute phase proteins			
CRP (mg/l), median [IQR][1,2]	2.10 [0.83-5.28]	2.80 [1.10-6.95]	1.45 [0.60-3.05]
AAT (mg/ml), median [IQR][3]	1.40 [1.20-1.70]	1.40 [1.20-1.70]	1.40 [1.20-1.65]
A2Macro (mg/ml),median [IQR][3]	1.50 [1.40-1.80]	1.50 [1.30-1.80]	1.60 [1.40-1.80]
Complement C3 (mg/ml), median [IQR][3]	0.90 [0.78-1.10]	0.90 [0.78-1.10]	0.92 [0.79-1.00]
Ferritin (mg/ml), median [IQR][1]	173.50 [94.75-283.0]	191.0 [102.50-320.50]	144.0 [82.0-242.50]
Haptoglobin (mg/ml), median [IQR][1,2]	1.40 [0.93-2.10]	1.50 [0.99-2.20]	1.30 [0.86-1.90]
PAI 1(ng/ml), median [IQR][1]	35.0 [23.0-53.0]	38.0 [27.0-61.50]	31.0 [21.0-46.0]
Fibrinogen (pg/ml), median [IQR][1]	3.50 [2.90-4.40]	3.60 [3.0-4.53]	3.40 [2.80-4.30]
Chemokine			
MIP-1 α (pg/ml), median [IQR][4]	16.0 [12.0-21.90]	15.0 [12.0-21.90]	17.0 [12.0-21.92]
MIP-1 β (pg/ml), median [IQR][1,2]	123.0 [92.0-165.0]	129.50 [95.0-177.25]	114.0 [89.45-146.0]
MCP-1 (pg/ml), median [IQR][1,2]	91.0 [70.0-121.75]	92.0 [70.0-133.0]	88.0 [70.50-111.0]
T-Cell-Specific RANTES (ng/ml), median [IQR][1]	11.0 [6.38-19.0]	14.0 [7.55-23.0]	9.06 [5.0-14.25]
Cytokines			
TNF-α (pg/ml), median [IQR][5]	1.95 [1.40-2.90]	1.80 [1.40-2.60]	2.0 [1.40-3.25]
TNF-β (pg/ml), median [IQR][6]	35.0 [18.0-116.0]	20.50 [16.50-44.25]	36.5 [27.0-152.75]
TNF R2 (ng/ml), median [IQR][1,2]	4.50 [3.60-5.71]	4.40 [3.50-5.80]	4.50 [3.65-5.64]
IFN-γ (pg/ml), median [IQR][3]	5.10 [3.85-7.30]	4.80 [3.80-6.60]	5.70 [4.20-8.23]
IL-6 (pg/ml), median [IQR][7]	3.50 [2.20-5.90]	3.70 [2.50-6.79]	2.50 [2.13-4.07]
IL-8 (pg/ml), median [IQR][1,2]	9.00 [6.80-12.0]	9.90 [7.30-13.0]	8.34 [6.53-10.83]
IL-10 (pg/ml), median [IQR][3]	5.20 [3.63-9.40]	6.90 [4.10-15.0]	4.50 [3.0-6.0]
IL-18 (pg/ml), median [IQR][3]	171.0 [132.50-215.0]	173.0 [134.0-217.0]	169.50 [130.50-211.25]
Renal markers			
Creatinine (umol/l), median [IQR][1,2]	77 [66.0-86.50]	77.0 [65.0-87.0]	76.0 [67.0-86.0]
Cystatin C (mg/ml), median [IQR][1]	796.0 [691.0-923.0]	791.0 [674.5-915.5]	802.0 [712.50-935.50]
NGAL (pg/ml), median [IQR][8]	201.0 [148.0-260.0]	207.0 [149.0-282.50]	186.0 [143.0-242.75]
Other markers			
Adiponectin, median [IQR][1]	2.80 [1.90-4.00]	2.80 [1.88-4.10]	2.86 [1.90-3.90]
Myoglobin (pg/ml), median [IQR][1,2]	38.0 [22.0-79.0]	51.0 [23.0- 190.0]	31.0 [20.0-50.50]
B2M (pg/ml), median [IQR][3]	1.30 [1.10-1.70]	1.30 [1.0-1.70]	1.40 [1.20-1.70]

All biomarkers are presented as median [IQR].
AAT, Alpha-1-Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha-2-Macroglobulin; B2M, beta-2-Microglobulin; CRP, C–reactive protein; IFN-ɣ, Interferon ɣ; IL, interleukin; IQR, interquartile range; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF-α, Tumor Necrosis Factor alpha; TNF-β, Tumor Necrosis Factor beta; TNF R2,Tumor Necrosis Factor Receptor2.

1 Blood samples available in 570 patients.
2 Measurable in 99% of 570 patients, too low to detect in 1%.
3 Blood samples available in 473 patients.
4 MIP-1 α was measurable in 84% of 570 patients, too low to detect in 16%
5 TNF- α was measurable in 92% of 473 patients, too low to detect in 8%.
6 TNF-β was measurable in 8% of 473 patients, too low to detect in 92%.
7 IL-6 was measurable in 38% of 570 patients, too low to detect in 62%.
8 NGAL was measurable in 97% of 473 patients, too low to detect in 3%.

Supplemental Table 4a. Univariate association between biomarkers with major adverse cardiac events (MACE; composite of all-cause mortality, nonfatal ACS or unplanned coronary revascularization) in the full cohort (n=570)

	Total (n=570)		ACS patients (n=309)		SAP patients (n=261)	
	HR [95% CI]	P-value	HR [95% CI]	P-value	HR [95% CI]	P-value
Acute phase proteins						
CRP[25]	1.05 (0.89-1.24)	0.56	1.10 (0.88-1.38)	0.41	1.18 (0.92-1.50)	0.19
AAT[26]	1.00 (0.63-1.58)	0.99	1.03 (0.53-2.00)	0.93	1.06 (0.56-2.02)	0.86
A2Macro[2]	1.91 (0.87-4.24)	0.11	1.53 (0.47-4.95)	0.48	1.84 (0.58-5.84)	0.30
Complement C3[2]	0.74 (0.31-1.78)	0.51	0.56 (0.15-2.14)	0.40	0.98 (031-3.13)	0.98
Ferritin[1]	0.94 (0.79-1.12)	0.50	1.20 (0.88-1.62)	0.25	0.89 (0.72-1.09)	0.26
Haptoglobin[1]	1.11 (0.72-1.69)	0.64	1.16 (0.62-2.16)	0.64	1.34 (0.74-2.44)	0.34
PAI 1[1]	0.91 (0.70-1.18)	0.47	0.91 (0.61-1.35)	0.62	1.08 (0.77-1.52)	0.66
Fibrinogen[1]	1.23 (0.75-2.01)	0.42	1.30 (0.63-2.68)	0.48	1.35 (0.68-2.69)	0.39
Chemokine						
MIP-1 α[1]	0.96 (0.72-1.28)	0.78	0.85 (0.54-1.34)	0.49	1.01 (0.69-1.47)	0.96
MIP-1 β[1]	0.93 (0.67-1.30)	0.66	0.94 (0.58-1.54)	0.82	1.09 (0.68-1.75)	0.73
MCP-1[1]	1.09 (0.79-1.51)	0.61	0.92 (0.59-1.44)	0.72	1.75 (1.03-2.96)	0.04
T-Cell-Specific RANTES[1]	0.72 (0.58-0.90)	0.003	0.65 (0.47-0.92)	0.01	0.90 (0.67-1.21)	0.49
Cytokines						
TNF α[2,27]	1.16 (0.91-1.48)	0.23	1.09 (0.71-1.68)	0.70	1.14 (0.85-1.52)	0.39
TNF β[28]	1.31 (0.74-2.30)	0.36	1.22 (0.38-3.90)	0.73	1.12 (0.58-2.16)	0.74
TNFR 2[1]	1.47 (0.96-2.27)	0.08	1.16 (0.61-2.22)	0.65	1.78 (0.99-3.22)	0.06
IFN-ɣ[2]	1.79 (1.28- 2.51)	0.001	1.88 (1.09-3.25)	0.02	1.56(1.00- 2.44)	0.05
IL-6[4]	0.93(0.67-1.29)	0.67	0.99 (0.61-1.61)	0.97	1.15 (0.72-1.84)	0.56
IL-8[1]	1.63 (1.21-2.20)	0.001	2.17 (1.47-3.20)	<0.001	1.39 (0.90-2.15)	0.14
IL-10[2]	1.00 (0.83-1.20)	0.98	0.99 (0.77-1.27)	0.93	1.45 (1.03-2.05)	0.03
IL-18[2]	0.82 (0.54-1.24)	0.35	0.74 (0.41-1.34)	0.32	0.97 (0.52-1.80)	0.92
Renal markers						
Creatinine[1,29]	1.11 (0.58-2.13)	0.76	0.67 (0.24-1.85)	0.44	1.81 (0.74-4.41)	0.19
Cystatin C[1]	1.91 (1.04-3.53)	0.04	1.97 (0.79-4.90)	0.15	1.78 (0.77-4.13)	0.18
NGAL[2]	1.37 (0.91-2.05)	0.13	1.94 (1.01-3.51)	0.03	1.16 (0.65-2.06)	0.61
Other markers						
Adiponectin[1]	1.09 (0.83-1.43)	0.54	1.79(1.18-2.72)	0.01	0.78 (0.56-1.10)	0.16
Myoglobin[1]	1.04 (0.92-1.18)	0.53	1.20 (1.02-1.40)	0.03	1.05 (0.78-1.31)	0.91
B2M[2]	1.81 (1.02-3.21)	0.04	1.25 (0.51-3.10)	0.63	2.17 (1.05-4.52)	0.04

Results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) biomarker concentration or per category of biomarker concentration, with 95 % confidence intervals (CI).
AAT, Alpha- 1- Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha- 2- Macroglobulin; B2M, beta-2-Microglobulin; CRP, C –reactive protein; IFN-ɣ, Interferon ɣ; IL, interleukin; IQR, interquartile range; MACE, major adverse cardiac events; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF α, Tumor Necrosis Factor alpha; TNF β, Tumor Necrosis Factor beta; TNFR 2,Tumor Necrosis Factor Receptor2.

25 Available in 570 patients.
26 Available in 473 patients.
27 Measurable in 92% of 473 patients, too low to detect in 8%.
28 Too low to detect in in a large part of the patients (TNF-β was measurable in 8% and IL 6 in 38% of the patients) and thus these biomarkers were not examined as continuous variables but as categorical variables (measurable vs not measurable).
29 Measurable in 99 % of 570 patients, too low to detect in 1 %.

Supplemental Table 4b. Multivariable adjusted association between biomarkers with major adverse cardiac events (MACE; composite of all-cause mortality, nonfatal ACS or unplanned coronary revascularization) in the full cohort (n=570)

	Total (n=570)		ACS patients (n=309)		SAP patients (n=261)	
	HR [95% CI][30]	P-value	HR [95% CI][31]	P-value	HR [95% CI][2]	P-value
Acute phase proteins						
CRP[32]	1.09 (0.92-1.29)	0.35	1.03 (0.81-1.30)	0.82	1.16 (0.90-1.50)	0.24
AAT[33]	0.99 (0.61-1.59)	0.95	0.88 (0.44-1.78)	0.73	1.05 (0.54-2.06)	0.88
A2Macro[4]	1.37 (0.59-3.17)	0.47	1.21 (0.37-4.00)	0.75	1.58 (0.48-5.27)	0.45
Complement C3[4]	0.72 (0.29-1.74)	0.46	0.52 (0.13-2.06)	0.35	0.96 (0.29-3.16)	0.94
Ferritin[3]	0.97 (0.82-1.16)	0.77	1.25 (0.92-1.71)	0.15	0.87 (0.70-1.07)	0.18
Haptoglobin[3]	1.16 (0.74-1.81)	0.52	1.01 (0.53-1.92)	0.98	1.31 (0.70-2.44)	0.40
PAI 1[3]	1.03 (0.80-1.33)	0.83	0.99 (0.66-1.49)	0.96	1.05 (0.75-1.47)	0.77
Fibrinogen[3]	1.12 (0.66-1.90)	0.67	0.96 (0.43-2.17)	0.96	1.25 (0.61-2.56)	0.55
Chemokine						
MIP-1 α[3,34]	0.89 (0.66-1.20)	0.44	0.71 (0.44-1.17)	0.19	1.01 (0.69-1.48)	0.97
MIP-1 β[3]	0.99 (0.70-1.40)	0.96	0.87(0.53-1.44)	0.60	1.09 (0.67-1.78)	0.73
MCP-1[3]	1.13 (0.79-1.60)	0.51	0.87 (0.54-1.40)	0.58	1.64 (0.95-2.81)	0.07
T-Cell-Specific RANTES[3]	0.82 (0.65-1.02)	0.07	0.71 (0.50-1.00)	0.05	0.91 (0.68-1.21)	0.50
Cytokines						
TNF α[4,35]	1.07 (0.84-1.37)	0.59	1.06(0.68-1.64)	0.81	1.10 (0.81-1.49)	0.54
TNF β[36]	1.14 (0.64- 2.03)	0.66	1.20 (0.37-3.88)	0.76	1.11 (0.57-2.16)	0.76
TNFR 2[3]	1.16 (0.73-1.85)	0.52	0.73 (0.36-1.48)	0.38	1.68 (0.91-3.13)	0.10
IFN-γ[4]	1.57 (1.10- 2.23)	0.012	1.75 (0.99-3.12)	0.06	1.51 (0.96- 2.36)	0.08
IL-6[37]	1.06 (0.75-1.49)	0.74	0.97 (0.59-1.58)	0.89	1.14 (0.70-1.84)	0.59
IL-8[3]	1.60 (1.18-2.17)	0.002	1.85 (1.22-2.80)	0.004	1.30 (0.82-2.01)	0.26
IL-10[4]	1.11 (0.91-1.35)	0.32	0.99 (0.76-1.28)	0.91	1.44 (1.02-2.04)	0.04
IL-18[4]	0.80 (0.52-1.22)	0.29	0.74 (0.41-1.33)	0.31	0.87 (0.47-1.63)	0.66
Renal markers						
Creatinine[38]	0.83 (0.40-1.72)	0.61	0.39 (0.13-1.23)	0.11	1.59 (0.56-4.55)	0.39
Cystatin C[3]	1.36 (0.69-2.65)	0.38	1.03 (0.36-2.93)	0.96	1.75 (0.71-4.33)	0.23
NGAL[4]	1.46 (0.96-2.21)	0.08	1.80 (1.01-3.22)	0.05	1.15 (0.64-2.07)	0.65
Other markers						
Adiponectin[3]	1.12 (0.82-1.52)	0.47	1.72 (1.07-2.76)	0.03	0.85 (0.58-1.25)	0.41
Myoglobin[3]	1.13 (0.98-1.31)	0.09	1.23 (1.04-1.46)	0.02	0.98 (0.74-1.30)	0.98
B2M[4]	1.41 (0.74-2.67)	0.30	0.68 (0.23-1.98)	0.48	2.33 (1.03-5.28)	0.04

Results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) biomarker concentration or per category of biomarker concentration, with 95 % confidence intervals (CI).
AAT, Alpha- 1- Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha- 2- Macroglobulin; B2M, beta-2-Microglobulin; CRP, C –reactive protein; IFN-γ, Interferon γ; IL, interleukin; IQR, interquartile range; MACE, major adverse cardiac events; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF α, Tumor Necrosis Factor alpha; TNF β, Tumor Necrosis Factor beta; TNFR 2,Tumor Necrosis Factor Receptor2.

30 Model is adjusted for age, gender, diabetes mellitus, hypertension, hypercholesterolemia and indication for coronary angiography.
31 Models are adjusted for age, gender, diabetes mellitus, hypertension and hypercholesterolemia.
32 Available in 570 patients.
33 Available in 473 patients.
34 Measurable in 84% of 570 patients, too low to detect in 16%
35 Measurable in 92% of 473 patients, too low to detect in 8%.
36 Too low to detect in a large part of the patients (TNF β measurable in 8% of 473 patients, too low to detect in 92), and thus these biomarkers were not examined as continuous variables but as categorical variables (measurable vs not measurable).
37 Too low to detect in a large part of the patients (IL-6 measurable in 38% of 570 patients, too low to detect in 62%), and thus these biomarkers were not examined as continuous variables but as categorical variables (measurable vs not measurable).
38 Measurable in 99% of 570 patients, too low to detect in 1%.

Supplemental Table 5. Multivariable adjusted association between biomarkers with the composite of all-cause mortality or nonfatal ACS (secondary endpoint) in the full cohort (n=570)

	Total (n=570)		ACS patients (n=309)		SAP patients (n=261)	
	HR [95% CI][39]	P-value	HR [95% CI][40]	P-value	HR [95% CI][2]	P-value
Acute phase proteins						
CRP[41]	1.27 (1.05-1.54)	0.02	1.24 (0.96-1.58)	0.10	1.31 (0.95-1.79)	0.10
AAT[42]	1.49 (0.87-2.55)	0.14	1.22 (0.59-2.52)	0.60	1.95 (0.86-4.45)	0.11
A2Macro[4]	1.93 (0.70-5.35)	0.21	1.83 (0.49-6.84)	0.37	2.26 (0.43-11.78)	0.33
Complement C3[4]	1.34 (0.46-3.89)	0.59	0.87 (0.19-3.92)	0.86	2.20 (0.47-10.23)	0.32
Ferritin[3]	1.04 (0.83-1.30)	0.76	1.47 (1.04-2.09)	0.03	0.80 (0.60-1.07)	0.13
Haptoglobin[3]	1.41 (0.82- 2.43)	0.21	1.23 (0.60-2.53)	0.58	1.63 (0.71- 3.72)	0.25
PAI 1[3]	1.27 (0.93-1.75)	0.13	1.16 (0.73-1.84)	0.53	1.45 (0.93-2.26)	0.10
Fibrinogen[3]	1.75 (0.92-3.31)	0.09	1.15 (0.62-3.67)	0.36	1.98 (0.76-5.16)	0.16
Chemokine						
MIP-1 α[3,4][43]	0.91 (0.63-1.32)	0.63	0.86 (0.50-1.48)	0.85	0.95 (0.57-1.56)	0.83
MIP-1 β[3]	0.87 (0.57-1.35)	0.54	1.08 (0.61-1.88)	0.80	0.64 (0.32-1.26)	0.20
MCP-1[3]	0.91 (0.69-1.57)	0.66	0.87 (0.51-1.50)	0.62	1.00 (0.47-2.09)	0.99
T-Cell-Specific RANTES[3]	0.84 (0.64-1.10)	0.19	0.67 (0.45-0.98)	0.039	1.07 (0.73-1.56)	0.73
Cytokines						
TNF α[4,44]	1.00 (0.72-1.38)	0.99	1.17 (0.73-1.88)	0.53	0.88 (0.57-1.37)	0.58
TNF β[4][45]	1.56 (0.80-3.03)	0.19	1.69 (0.52-5.51)	0.38	1.41 (0.62-3.18)	0.41
TNFR 2[3]	1.23 (0.69-2.18)	0.48	1.10 (0.50-2.43)	0.81	1.41 (0.61-3.29)	0.43
IFN-ɣ[4]	1.58 (1.03- 2.43)	0.04	1.68 (0.89-3.18)	0.11	1.54 (0.85-2.78)	0.16
IL-6[4][46]	1.25 (0.83-1.89)	0.28	1.63 (0.93-2.86)	0.09	0.86 (0.44-1.67)	0.66
IL-8[3]	1.75 (1.24-2.48)	0.0015	2.89 (1.86-4.14)	<0.001	1.12 (0.61-2.03)	0.72
IL-10[4]	1.14 (0.90-1.45)	0.27	1.10 (0.83-1.46)	0.52	1.35 (0.86-2.12)	0.20
IL-18[4]	0.90 (0.54-1.50)	0.70	0.88 (0.47-1.67)	0.70	0.97 (0.42-2.26)	0.95
Renal markers						
Creatinine [4][47]	0.85 (0.35-2.06)	0.71	0.56 (0.16-1.96)	0.36	1.49 (0.38-5.93)	0.57
Cystatin C[3]	1.83 (0.82-4.09)	0.14	2.05 (0.68-6.02)	0.20	1.68 (0.51-5.53)	0.39
NGAL[4]	1.44 (0.87-2.39)	0.16	2.66 (1.37-5.19)	0.004	0.60 (0.28-1.27)	0.18
Other markers						
Adiponectin[3]	1.18 (0.81-1.73)	0.39	2.03 (1.20-3.44)	0.008	0.69 (0.43-1.11)	0.12
Myoglobin[3]	1.14 (0.96-1.35)	0.13	1.23 (1.01-1.49)	0.04	1.02 (0.70-1.49)	0.90
B2M[4]	2.29 (1.04-5.06)	0.04	1.52 (0.48-4.83)	0.48	3.45 (1.14-10.44)	0.03

Results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) biomarker concentration or per category of biomarker concentration, with 95 % confidence intervals (CI).
AAT, Alpha- 1- Antitrypsin; ACS, acute coronary syndrome; A2Macro, alpha- 2- Macroglobulin; B2M, beta-2-Microglobulin; CRP, C –reactive protein; IFN-ɣ, Interferon ɣ; IL, interleukin; IQR, interquartile range; MACE, major adverse cardiac events; MCP-1, Monocyte Chemotactic Protein 1; MI, myocardial infarction; MIP-1α, Macrophage Inflammatory Protein-1 alpha; MIP-1β, Macrophage Inflammatory Protein-1 beta; NGAL, Neutrophil Gelatinase-Associated Lipocalin; PAI 1, Plasminogen Activator Inhibitor 1; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TNF α, Tumor Necrosis Factor alpha; TNF β, Tumor Necrosis Factor beta; TNFR 2,Tumor Necrosis Factor Receptor2.

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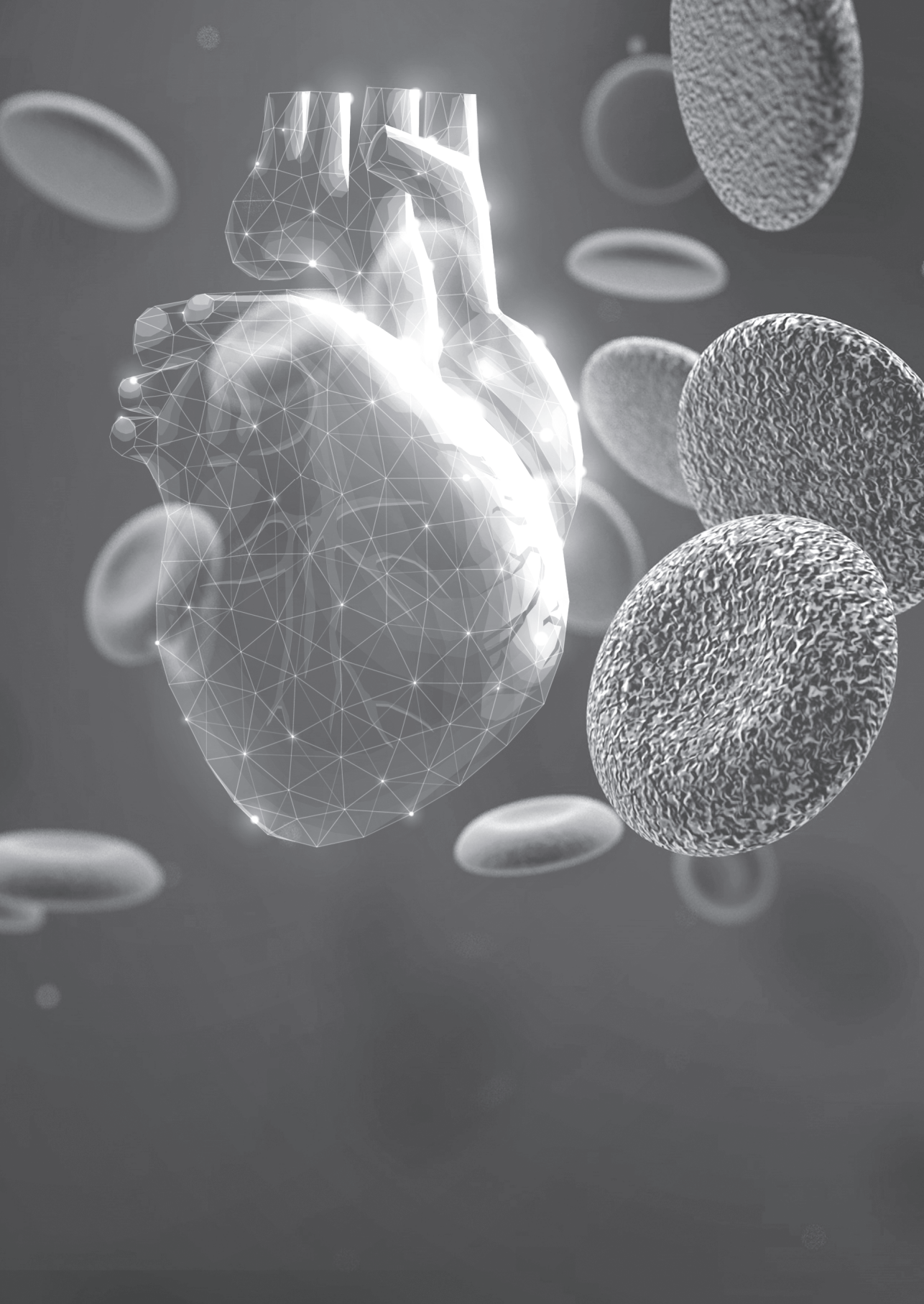
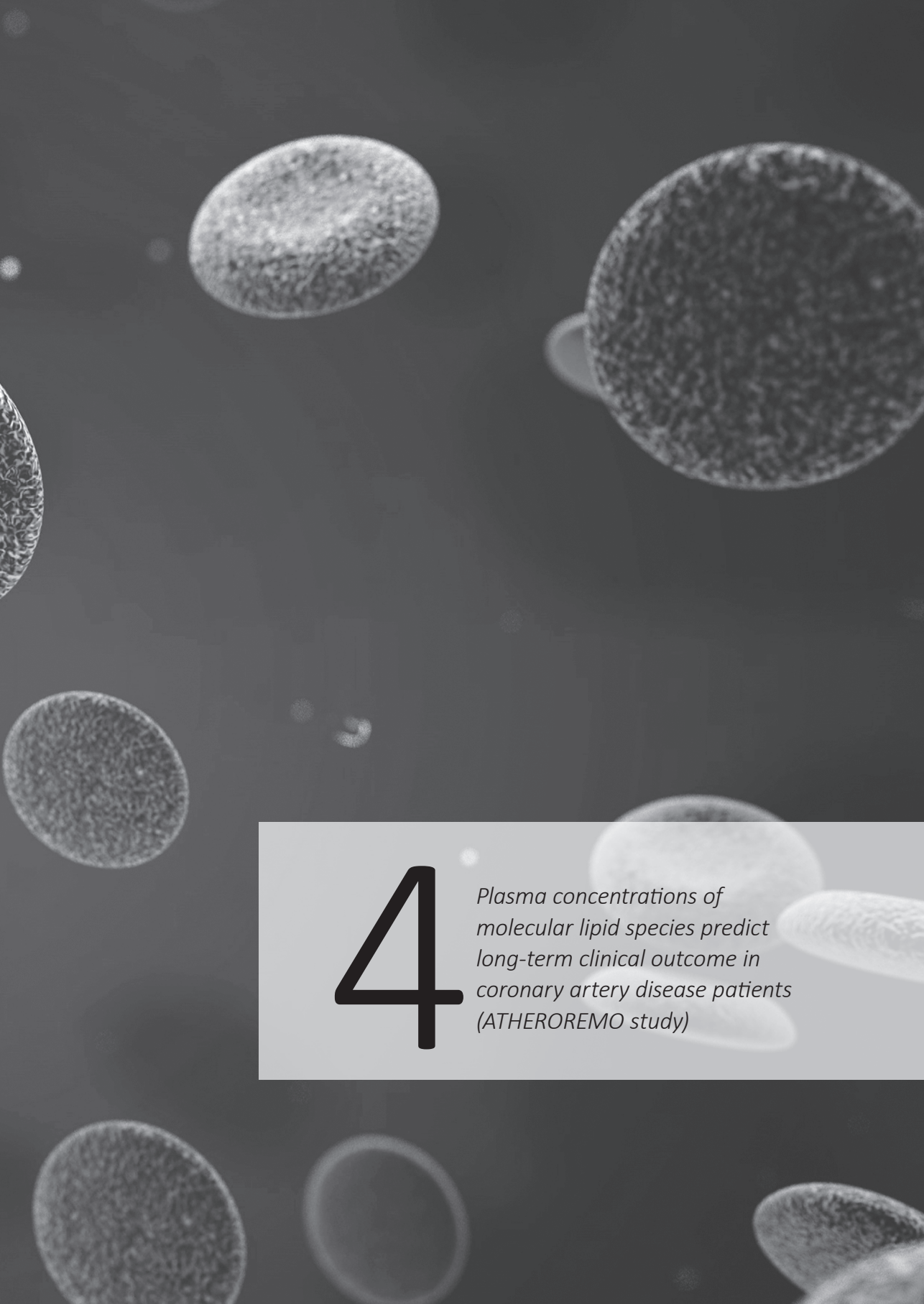
45 TNF β measurable in 8% of 473 patients, too low to detect in 92 %; thus these biomarkers were not examined as continuous variables but as categorical variables (measurable vs not measurable).
46 IL-6 measurable in 38% of 570 patients, too low to detect in 62 %; thus these biomarkers were not examined as continuous variables but as categorical variables (measurable vs not measurable).
47 Measurable in 99% of 570 patients, too low to detect in 1%.





4

Plasma concentrations of molecular lipid species predict long-term clinical outcome in coronary artery disease patients (ATHEROREMO study)

4

Plasma concentrations of molecular lipid species predict long-term clinical outcome in coronary artery disease patients (ATHEROREMO study)

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ABSTRACT

Purpose: We investigated the associations of ten previously identified high risk molecular lipid species and three ceramide ratios with the occurrence of major adverse cardiac events (MACE) during a median follow-up of 4.7 years in patients with coronary artery disease (CAD).

Methods: Between 2008 and 2011, 581 patients underwent diagnostic coronary angiography or percutaneous coronary intervention for stable angina pectoris (SAP) or acute coronary syndrome (ACS). Blood was drawn prior to the index procedure and lipid species were determined. The primary endpoint was the occurrence of MACE, comprising all-cause mortality, nonfatal ACS or unplanned coronary revascularization. The secondary endpoint comprised all-cause mortality or nonfatal ACS. Results: During a median follow-up of 4.7 [IQR: 4.2- 5.6] years, 155 patients (27%) had MACE. In multivariable analyses, Cer(d18:1/16:0) concentration was associated with MACE (HR 2.32; 95% CI [1.09-4.96] per ln(pmol/mL) p= 0.030) after adjustment for cardiac risk factors, clinical presentation, statin use at baseline and admission non-HDL cholesterol level. Furthermore, after multivariableadjustment, concentrations of Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:1) and their ratios to Cer(d18:1/24:0) were associated with the composite endpoint death or nonfatal ACS.

Conclusion: Altogether, the circulating ceramide lipids we investigated here are associated with adverse cardiac outcome during long-term follow-up independent of clinical risk factors.

INTRODUCTION

Established lipid markers such as total cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG) and high-density lipoprotein (HDL) cholesterol have long formed the cornerstone of lipid-based risk stratification in coronary artery disease (CAD) (1-4). However these measures alone do not fully capture the complexity of the altered lipid metabolism in cardiovascular disease (2), and this may be the reason that they fail to identify a substantial proportion of patients at high risk for coronary events (1).

Lipidomics is a systems-based study of all lipids (5) that has been defined as the full characterization of lipid molecular species and their biological roles (6). In its most advanced form, lipidomics is able to quantify hundreds of diverse molecular lipid species across multiple lipid classes such as sphingolipids, phospholipids, sterol esters, and acylglycerols, (7) many of which play an integral role in modulation of biological function such as formation of cellular membranes, energy storage and cell signaling (8, 9). Since lipidomics provides such detailed lipid profiles, it may further improve risk stratification of CAD patients and provide novel mechanistic insights into CAD (4).

In line with this hypothesis, we have recently performed lipidomics in the Ludwigshafen Risk and Cardiovascular Health (LURIC) study and identified several molecular lipid species that are associated with fatal events in patients with CAD (1). In the current study, we hypothesized that these ten previously identified high risk molecular lipid species and three ceramide ratios are associated with occurrence of major adverse cardiac events (MACE) during long-term follow-up.

METHODS

Study population and design

The design of the European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis (ATHEROREMO) has been described elsewhere in detail (10). In brief, from 2008 until 2011, 581 patients with an indication for diagnostic coronary angiography (CAG) and/or percutaneous coronary intervention (PCI) due to stable angina pectoris (SAP) or acute coronary syndrome (ACS) were included at the Erasmus MC, Rotterdam, the Netherlands. Prior to the CAG or PCI procedure blood samples were collected from the arterial sheath and were transported to the clinical laboratory of Erasmus MC within 2h after blood collection for storage at a temperature of −80 °C. All included patients were 18 years or older. The

ATHEROREMO study was approved by the medical ethics committee of Erasmus MC and was performed in accordance with the criteria described in the declaration of Helsinki. Written informed consent was obtained from all included patients.

Serum concentrations of cholesterol and triglycerides

Levels of total cholesterol, LDL cholesterol, HDL cholesterol and TG were measured in the clinical laboratory of the Erasmus MC in serum samples using Roche/Hitachi cobas c 701/702 analyzer (Roche Diagnostics, Indianapolis, USA) on the Cobas 8000 modular analyzer platform (Roche Diagnostics, Indianapolis, USA).

Plasma concentrations of molecular lipids

Molecular lipids and lipid ratios that were previously found to be associated with fatal cardiovascular outcome at a $p < 0.05$ level in the LURIC study were selected for evaluation in the current study(1). These included cholesteryl esters (CE): CE 14:0, CE 18:3, CE 20:4, CE 20:5, CE 22:5; ceramides (Cer): Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:0), Cer(d18:1/24:1); ceramide ratios Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/20:0)/Cer(d18:1/24:0), Cer(d18:1/24:1)/Cer(d18:1/24:0)), and lactosylceramide (LacCer): LacCer(d18:1/18:0).

Plasma samples for measurement of lipid concentrations were available in 574 patients. Stored plasma samples were subjected to lipid extraction at Zora Biosciences, Finland. Briefly, Samples (10 µL) were spiked with known amounts of lipid-class specific, non-endogenous synthetic internal standards, D6-CE 18:0 (C/D/N Isotopes Inc.,Pointe-Claire, Quebec, Canada), Cer(d18:1/17:0) (Avanti Polar Lipids Inc., Alabaster, AL, USA) and D3-LacCer(d18:1/16:0) (Matreya LLC, State College, Pennsylvania, USA). Lipid extraction was performed using chloroform (HPLC grade) (Rathburn Chemicals Ltd., Walkerburn, Scotland), methanol, and acetic acid (both LC-MS grade) (Sigma-Aldrich GmbH, Steinheim, Germany) (11). After lipid extraction, samples were reconstituted in chloroform: methanol (1:2, v/v) for sphingolipids analysis, and for molecular shotgun lipidomic analysis the extracts were further diluted with chloroform/methanol (1:2, v/v) containing 5 mM ammonium acetate. Quality control samples (QC) were prepared along with the actual samples for lipidomic analyses to monitor the extraction and MS performance. The intra-day (n=3) average coefficient of variation (CV) of sphingolipids and CE was less than or equal to 6% and inter-day (n=24 for Cer and LacCer; n=23 for CE except for CE(22:5) n=22) CV was less than 21% for both sphingolipids and CE.

Sphingolipids were analyzed on a QTRAP 5500 mass spectrometer (AB SCIEX, Concord, Canada)® equipped with an ultra-high pressure liquid chromatography (UHPLC) system CTC PAL autosampler (Leap Technologies) and Accela 1250 Pump (Thermo Fisher Scientific., Massachusetts, United States). Chromatographic separation was performed on an Acquity BEH C18, 2.1 × 50 mm column with a particle size of 1.7 µm (Waters, Milford, MA). Mobile phases were 10 mM ammonium acetate in water with 0.1% formic acid (solvent A) and 10 mM ammonium acetate in acetonitrile:isopropanol (4:3, v/v) containing 0.1% formic acid (solvent B). Lipids were separated with linear gradient from 75% B to 100% B in 15 min. Flow rate was 500 µL/min and column temperature was 60°C. Data was collected using multiple reaction monitoring in positive ion mode (12). Curtain gas was set at 25, ion spray voltage was set at 5000 and ion source was heated to 400°C. Collision energy was optimized for each lipid class. Collision energy for Cer and LacCer was set to 40 and 45, respectively.

Shotgun lipidomics was performed to monitor CE on a QTRAP 5500 mass spectrometer (AB® SCIEX, Concord, Canada) equipped with a robotic nanoflow ion source NanoMate HD (Advion, NY, USA) as described (11). CE were analyzed in positive ion mode using precursor ion scanning (PIS) of 369.35 with collision energy 30 (13). Mass spectrometry data files were processed using MultiQuant 2.0.1 or ™LipidView 1.0 (AB SCIEX, Concord, Canada) (13). Identified lipids were quantified by normalizing against ™ their respective internal standard and volume of plasma used for the extraction. The limit of quantification (LOQ) for Cer, LacCer and CE in extract was 0.0004 µM, 0.0016 µM and 0.012 µM, respectively. All lipids monitored were within the LOQ. The LOQ was defined as the lowest point in the calibration curve with a signal-to-noise ratio greater than or equal to 10.

Follow-up and study endpoints

Clinical and vital status of patients were collected from medical charts, civil registries or by written or telephone contacts with the patients or relatives. All living patients participating in this study received a questionnaire, consisting of queries regarding the occurrence of MACE and re-admissions. For patients with adverse events, hospital discharge letters were obtained and treating physicians or institutions were contacted if necessary for additional information.

The primary endpoint was the occurrence of MACE, comprising all-cause mortality, nonfatal ACS or unplanned coronary revascularization. The secondary endpoint comprised all-cause mortality and nonfatal ACS. ACS was defined as the clinical

diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris (UAP) in accordance with the guidelines of the European society of Cardiology (14, 15). Unplanned coronary revascularization was defined as unplanned repeated PCI or unplanned coronary artery bypass grafting (CABG). The endpoints were adjudicated according to their definitions by a clinical events committee that was blinded to the lipid data.

Statistical analysis

Categorical variables are presented as numbers and percentages. The distributions of continuous variables, including lipid concentrations and lipid ratios, were examined for normality by visual inspection of the histogram. Normally distributed continuous variables are presented as mean± standard deviation (SD). Non-normally distributed continuous variables (which included molecular lipid concentrations and lipid ratios) are presented as median (interquartile range [IQR]) and were logarithmically (Ln) transformed for further analyses. Patients lost during follow-up were considered at risk until the date of last contact, at which time-point they were censored.

Cox proportional hazards models were used to evaluate the associations between molecular lipids and clinical study endpoints. For patients who experienced more than 1 event, the first was considered. The results are presented as hazard ratios (HRs) per unit increase in (Ln- transformed) molecular lipid concentrations or lipid ratios, with 95% confidence intervals (CIs). First, all analyses were performed univariably. In the multivariable analyses, gender, age, hypertension, hypercholesterolemia, diabetes mellitus and statin use were considered as potential confounders and were entered as covariates. These covariates were chosen for etiologic reasons and were based on existing literature (16). To evaluate whether the associations between molecular lipids and the clinical endpoints are independent of serum LDL cholesterol levels or serum non-HDL cholesterol levels, baseline serum LDL cholesterol level and baseline serum non-HDL cholesterol level were additionally (and consecutively) added into the multivariable models. Serum non-HDL level was calculated by subtracting HDL cholesterol level from total cholesterol level. In the full cohort, indication for CAG (ACS versus SAP) was also entered as a covariate. Interaction terms were added to the model to account for possible effect modification by indication for baseline CAG. Subsequently, analyses were stratified on indication for CAG.

Table 1. Clinical characteristics

Clinical characteristics	Total (n=574)	ACS patients (n=313)	SAP patients (n= 261)	p-value
Age, years, mean ± SD	61.5 ± 11.3	59.7 ± 11.9	63.6 ± 10.3	<0.001
Male, n (%)	432 (75)	230 (74)	202 (77)	0.279
Diabetes Mellitus, n (%)	97 (17)	40 (13)	57 (22)	0.004
Hypertension, n (%)	298 (52)	137 (44)	161 (62)	<0.001
Hypercholesterolemia, n (%)	318 (55)	138 (44)	180 (69)	<0.001
Smoking, n (%)	166 (29)	116 (37)	50 (19)	<0.001
Positive family history of CAD, n (%)	298 (52)	145 (46)	153 (59)	0.004
Previous MI, n (%)	184 (32)	80 (26)	104 (40)	<0.001
Previous PCI, n (%)	184 (32)	57 (18)	127 (49)	<0.001
Previous CABG, n (%)	18 (3)	7 (2)	11 (4)	0.176
Previous stroke, n (%)	26 (5)	11 (4)	15 (6)	0.200
Peripheral artery disease, n (%)	35 (6)	11 (4)	24 (9)	0.005
History of heart failure, n (%)	19 (3)	6 (2)	13 (5)	0.041
Serum LDL cholesterol, mmol/L	2.71 [2.12-3.54]	3.10 [2.32-3.87]	2.37 [1.94-2.99]	<0.001
Serum HDL cholesterol, mmol/L	1.04 [0.87-1.29]	1.05 [0.87-1.27]	1.03 [0.86-1.30]	0.80
Serum TG mmol/L	1.27 [0.88-1.83]	1.15 [0.77-1.77]	1.41 [1.05-1.94]	<0.001
Statin use at baseline, n (%)	508 (89%)	308 (98%)	235 (90%)	0.499
Procedural characteristics				
Indication for CAG				
ACS, n (%)	313 (55)	313 (100)	0 (0)	
STEMI, n (%)	162 (28)	162 (52)	0 (0)	
Non-ST-elevation, n (%)	151 (26)	151 (48)	0 (0)	
Stable angina pectoris, n (%)	261 (46)	0 (0)	261 (100)	
PCI performed, n (%)	505 (88)	291 (93)	214 (82)	
Coronary artery disease *				
No significant stenosis, n (%)	42 (7)	18 (6)	24 (9)	
1-vessel disease, n (%)	304 (53)	172 (55)	132 (51)	
2-vessel disease, n (%)	167 (29)	88 (28)	79 (30)	
3-vessel disease, n (%)	61 (11)	35 (11)	26 (10)	

* A significant stenosis was defined as a stenosis ≥ 50% of the vessel diameter by visual assessment of the coronary angiogram.

Continuous variables are presented as mean± standard deviation (SD) or median [IQR]. Categorical variables are presented in numbers (n) and percentages (%).

P-value was obtained from student's t-test.

ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CAG, coronary angiography; HDL, high-density lipoprotein; IQR, interquartile range; LCBI, Lipid Core Burden Index; LDL, low-density lipoprotein; MI, myocardial infarction; PCI, percutaneous coronary intervention; SAP, stable angina pectoris; TG, triglycerides.

Table 2. Lipid concentrations in the full cohort, MACE cohort and non-MACE cohort

Lipid concentrations	Total (n=574)	ACS patients (n=313)	SAP patients (n= 261)	p-value
CE 14:0, pmol/µl	21.7 [15.9-28.1]	22.9 [16.5-30.5]	21.2 [15.4-26.8]	0.008
CE 18:3, pmol/µl	70.3 [51.8-90.7]	72.3 [53.6-99.5]	66.1 [50.3-85.2]	0.003
CE 20:4, pmol/µl	386 [317-457]	394 [324-453]	374 [307-471]	0.31
CE 20:5, pmol/µl	49.1 [36.3-72.6]	49.2 [36.4-72.1]	49.0 [35.9-74.7]	0.69
CE 22:5, pmol/µl	2.65 [2.00-3.62]	2.81 [2.12-3.77]	2.53 [1.90-3.40]	0.037
Cer(d18:1/16:0) pmol/µl	0.12 [0.10-0.15]	0.13 [0.11-0.17]	0.11 [0.09-0.13]	<0.001
Cer(d18:1/20:0) pmol/µl	0.11 [0.09-0.15]	0.12 [0.10-0.16]	0.11 [0.08-0.13]	<0.001
Cer(d18:1/24:0) pmol/µl	5.98 [4.72-7.49]	6.43 [5.00-8.07]	5.65 [4.49-6.61]	<0.001
Cer(d18:1/24:1) pmol/µl	1.79 [1.42-2.25]	1.89 [1.52-2.44]	1.67 [1.35-2.05]	<0.001
LacCer(d18:1/18:0) pmol/µl	0.13 [0.10-0.16]	0.13 [0.11-0.16]	0.12 [0.10-0.15]	0.001
Cer(d18:1/16:0)/Cer(d18:1/24:0) pmol/µl	0.020 [0.018-0.024]	0.021 [0.018-0.025]	0.020 [0.017-0.023]	0.001
Cer(d18:1/20:0)/Cer(d18:1/24:0) pmol/µl	0.019 [0.016-0.024]	0.019 [0.015-0.024]	0.019 [0.016-0.023]	0.62
Cer(d18:1/24:1)/Cer(d18:1/24:0) pmol/µl	0.31 [0.26-0.36]	0.31 [0.26-0.36]	0.31 [0.26-0.36]	0.65
Lipid concentrations in the MACE cohort	Total (n=155	ACS patients (n=65)	SAP patients (n= 90)	p-value
CE 14:0, pmol/µl	22.6 [15.7-27.1]	21.7 [15.5-30.3]	22.7 [15.7-26.6]	0.67
CE 18:3, pmol/µl	67.9 [51.2-90.3]	70.3 [52.8-103]	66.9 [50 -84.1]	0.17
CE 20:4, pmol/µl	381 [310-445]	381 [310-432]	379 [310-447]	0.95
CE 20:5, pmol/µl	52.4 [37.4-74.5]	52.6 [38.4-76.3]	50.9 [36.6-74.4]	0.73
CE 22:5, pmol/µl	2.57 [1.86-3.71]	2.61 [2.04-3.97]	2.51 [1.80-3.45]	0.065
Cer(d18:1/16:0) pmol/µl	0.12 [0.10-0.16]	0.15 [0.11-0.17]	0.11 [0.09-0.13]	<0.001
Cer(d18:1/20:0) pmol/µl	0.11 [0.09-0.16]	0.13 [0.10-0.17]	0.11 [0.08-0.14]	0.011
Cer(d18:1/24:0) pmol/µl	5.86 [4.65-7.48]	6.44 [5.02-8.10]	5.66 [4.54-6.58]	0.063
Cer(d18:1/24:1) pmol/µl	1.78 [1.35-2.36]	2.10 [1.66-2.84]	1.62 [1.33-2.13]	0.008
LacCer(d18:1/18:0) pmol/µl	0.13 [0.10-0.16]	0.14 [0.10-0.18]	0.13 [0.10-0.16]	0.137
Cer(d18:1/16:0)/Cer(d18:1/24:0) pmol/µl	0.021 [0.018-0.025]	0.022 [0.019-0.027]	0.019 [0.017-0.024]	0.006
Cer(d18:1/20:0)/Cer(d18:1/24:0) pmol/µl	0.020 [0.016-0.025]	0.020 [0.016-0.024]	0.020 [0.015-0.025]	0.504
Cer(d18:1/24:1)/Cer(d18:1/24:0) pmol/µl	0.31 [0.27-0.37]	0.33 [0.27-0.36]	0.31 [0.26-0.37]	0.222
Lipid concentrations in the non-MACE cohort**	Total (n=411)	ACS patients (n=242)	SAP patients (n= 169)	p-value
CE 14:0, pmol/µl	21.5 [15.8-28.7]	23 [16.5-30.5]	20.7 [15.3-27]	0.007
CE 18:3, pmol/µl	70.5 [51.7-91.3]	73.8 [53.5-99]	66 [50.3 -86.4]	0.011
CE 20:4, pmol/µl	391 [321-467]	397 [310-432]	374 [304-476]	0.272
CE 20:5, pmol/µl	49.1 [35.6-70.5]	49.2 [35.5-70.7]	47.5 [35.6-70]	0.575
CE 22:5, pmol/µl	2.69 [2.04-3.58]	2.79 [2.12-3.70]	2.54 [1.92-3.40]	0.041
Cer(d18:1/16:0) pmol/µl	0.12 [0.10-0.15]	0.13 [0.11-0.16]	0.11 [0.09-0.13]	<0.001
Cer(d18:1/20:0) pmol/µl	0.12 [0.09-0.14]	0.12 [0.09-0.15]	0.11 [0.08-0.13]	<0.001
Cer(d18:1/24:0) pmol/µl	6 [4.75-7.47]	6.39 [4.97-8.07]	5.64 [4.49-6.64]	<0.001

Cer(d18:1/24:1) pmol/µl	1.78 [1.35-2.36]	1.86 [1.51-2.33]	1.68 [1.35-2.04]	<0.001
LacCer(d18:1/18:0) pmol/µl	0.13 [0.10-0.16]	0.13 [0.11-0.16]	0.12 [0.10-0.15]	0.001
Cer(d18:1/16:0)/Cer(d18:1/24:0) pmol/µl	0.021 [0.018-0.025]	0.021 [0.018-0.024]	0.020 [0.017-0.023]	0.017
Cer(d18:1/20:0)/Cer(d18:1/24:0) pmol/µl	0.020 [0.016-0.025]	0.019 [0.015-0.024]	0.019 [0.016-0.023]	0.60
Cer(d18:1/24:1)/Cer(d18:1/24:0) pmol/µl	0.31 [0.27-0.37]	0.30 [0.25-0.36]	0.31 [0.26-0.36]	0.77

Concentrations are presented in µM as median [IQR]. P-value was obtained from student's t-test for difference in ln-transformed mean lipid concentration.
ACS, acute coronary syndrome; CE, cholesteryl ester; Cer, ceramide; LacCer, lactosylceramide; MACE, major adverse cardiac events SAP, stable angina pectoris.

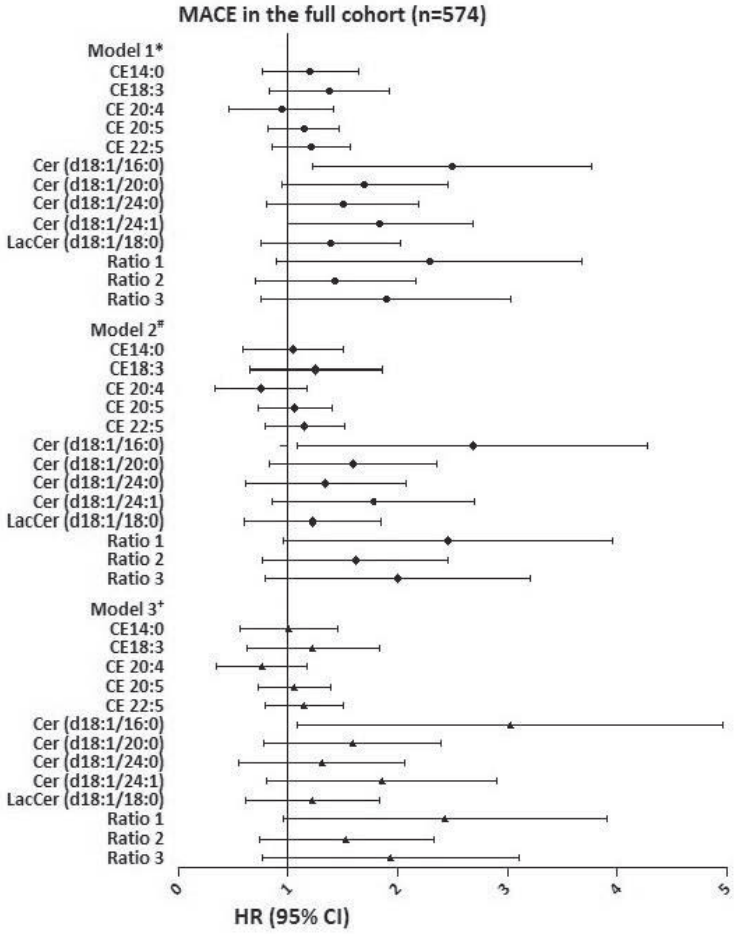
All data were analyzed with SPSS software (SPSS 23.0 IBM corp., Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics

The baseline clinical characteristics and the lipid concentrations of the ATHEROREMO study are summarized in Table 1 and Table 2. In total 574 patients were included. The mean age of the patients was 61.5 years and 75% were men. A total of 55% patients were diagnosed with ACS (28% STEMI and 26% non-STEMI), and 46% patients with SAP. PCI was performed in 88% of the patients during the index procedure. Prior to the index procedure median serum LDL cholesterol level was 2.71 [IQR: 2.12- 3.54] mmol/l, median serum HDL cholesterol level was 1.04 [IQR: 0.87- 1.29] mmol/l, median serum non-HDL cholesterol level was 3.23 [2.54- 4.00] mmol/l, and median serum triglyceride (TG) level was 1.27 [IQR: 0.88- 1.83] mmol/l in the full cohort. ACS patients had significantly higher serum LDL cholesterol level ((median: 3.10 [IQR: 2.32- 3.87] mmol/l) p= < 0.001), higher serum non-HDL cholesterol level ((median: 3.56 [2.81- 4.36]mmol/l) p = < 0.001) and lower serum TG level ((median= 1.15 [IQR: 0.77- 1.77] mmol/l) p = < 0.001)) compared with SAP patients (median: 2.37 [IQR: 1.94- 2.99] mmol/l, 2.83 [2.35- 3.56] mmol/l and 1.41 [IQR: 1.05- 1.94] mmol/l, respectively). In addition, several other clinical characteristics were significantly different between the ACS patients and the SAP patients (Table 1). At the time of hospital admission 89% of the patients in the full cohort used statins.

As shown in Table 2, ACS patients had significantly higher plasma concentrations of CE 22:5; Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:0), Cer(d18:1/24:1),



* Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus and statin use.

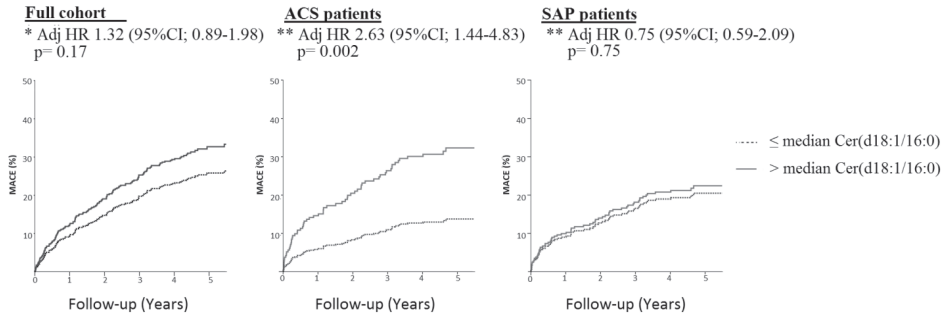
Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, statin use and serum LDL cholestero level at baseline.

+ Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, statin use and serum non-HDL cholesterol level at baseline.

Ratio 1:Cer(d18:1/16:0)/Cer(d18:1/24:0)
Ratio 2:Cer(d18:1/20:0)/Cer(d18:1/24:0)
Ratio 3:Cer(d18:1/24:1)/Cer(d18:1/24:0)

Figure 1. Association of plasma concentrations of molecular lipid species with MACE.
The results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) molecular lipid concentrations or lipid ratios, with 95% confidence intervals (CI).
CE, cholesteryl ester; Cer, ceramide; LacCer, lactosylceramide; MACE, major adverse cardiac events

LacCer(d18:1/18:0) and Cer(d18:1/16:0)/Cer(d18:1/24:0) in the full cohort and



* Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation, statin use and serum non-HDL cholesterol levels at baseline.
** Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, statin use and serum non-HDL cholesterol levels at baseline.

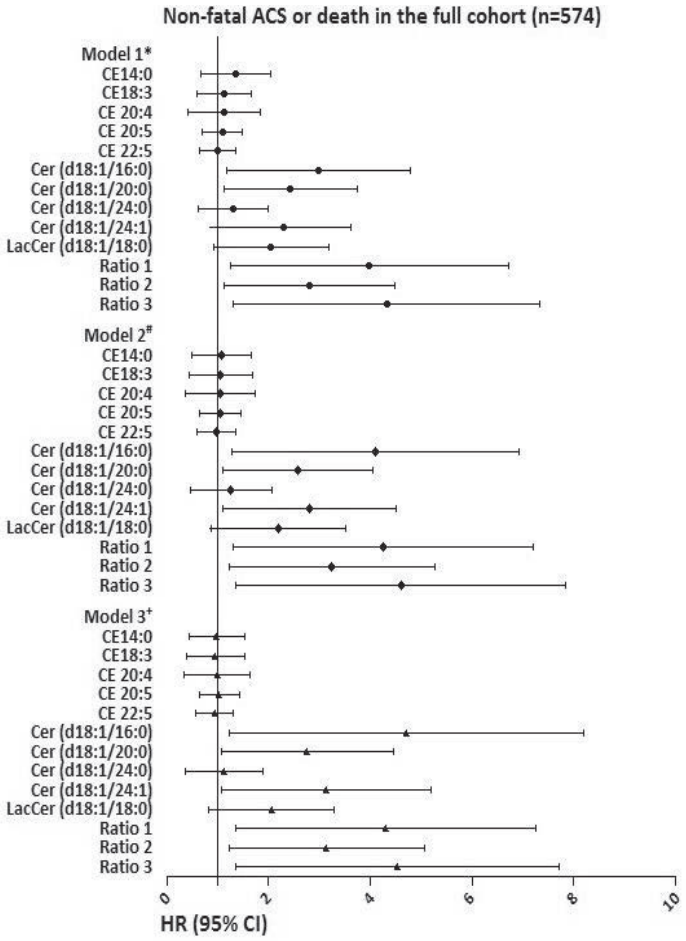
Figure 2. Association of plasma concentrations of Cer (d18:1/16:0) with MACE in the full cohort and in patients with ACS or SAP.
The results are presented as Hazard Ratios (HRs) for Cer(d18:1/16:0) above versus below the median, with 95% confidence intervals (CI).
ACS, acute coronary syndrome; Cer, ceramide; MACE, major adverse cardiac events; SAP, stable angina pectoris.

in patients with no event (the non-MACE cohort) as compared with SAP patients. In patients with an event (MACE cohort), except plasma concentration of LacCer(d18:1/18:0), all of the above mentioned lipid species plasma concentrations were significantly higher in the ACS patients as compared with SAP patients. In addition, in ACS patients concentration of Cer(d18:1/16:0) was significant higher (p=0.054) in the MACE cohort as compared with the non-MACE cohort.

Molecular lipids concentrations and cardiovascular outcome

In the full cohort (n=574) vital status was acquired for 572 patients (99.7%). The follow-up questionnaire assessing the occurrence of MACE was completed by 99% of the 574 patients. During a median follow-up time of 4.7 years (IQR: [4.2- 5.6]) years, a total of 155 patients (27%) experienced at least 1 MACE (primary endpoint). In the ACS group, 65 patients (21%) experienced MACE during long- term follow-up, in the SAP group this was 90 patients (34%).

The results for the associations between the molecular lipids concentrations and MACE are depicted in Figure 1 and Supplemental Table S1a. In multivariable analyses, after adjustment for cardiac risk factors, clinical presentation and statin use at baseline, Cer(d18:1/16:0) concentration (HR: 2.14; 95% CI [1.22- 3.76] per ln(pmol/mL) p= 0.008) and Cer(d18:1/24:1) concentration (HR: 1.64; 95% CI



* Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation and statin use.

Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation, statin use and serum LDL cholesterol level at baseline.

+ Adjusted for gender, age, hypertension, hypercholesterolemia, diabetes mellitus, clinical presentation, statin use and serum non-HDL cholesterol level at baseline.

Ratio 1: Cer (d18:1/16:0)/ Cer (d18:1/24:0)
Ratio 2:Cer (d18:1/20:0)/ Cer (d18:1/24:0)
Ratio 3:Cer (d18:1/24:1)/ Cer (d18:1/24:0)

Figure 3. Association of plasma concentrations of molecular lipid species with non-fatal ACS or death.

The results are presented as hazard ratios (HRs) per unit increase in (Ln-transformed) molecular lipid concentrations or lipid ratios, with 95% confidence intervals (CI).

ACS, acute coronary syndrome CE, cholesteryl ester; Cer, ceramide; LacCer, lactosylceramide.

[1.00- 2.68] per ln(pmol/mL) p= 0.049) were significantly associated with MACE. After additional adjustment for admission serum LDL cholesterol level, or serum

non-HDL cholesterol level, only Cer(d18:1/16:0) concentration remained associated with MACE (HR: 2.16; 95% CI [1.09- 4.27] per ln(pmol/mL), p=0.027, and HR: 2.32; 95% CI [1.09- 4.96] per ln(pmol/mL, p= 0.030, respectively).

The interaction term between Cer(d18:1/16:0) and indication for CAG was significant (p= 0.030) in the multivariable model. In ACS patients, higher Cer(d18:1/16:0) concentration was significantly associated with MACE, both in the uni- and multivariable model (HR adjusted for cardiac risk factors, statin use and non-HDL cholesterol level at baseline: 6.13; 95% CI [1.65 - 22.8]) per ln(pmol/mL) p= 0.007) (Supplemental Table S2a). In SAP patients, HRs were closer to the null and did not reach statistical significance (Supplemental Table S2b). The interaction term between Cer(d18:1/16:0)/Cer(d18:1/24:0) and indication for CAG was also significant (p= 0.044). Its association with MACE only reached statistical significance, in univariable analysis in ACS patients (Supplemental table S2a).

The incidence of MACE for Cer(d18:1/16:0) levels above and below the median are depicted in Figure 2. After adjustment for cardiac risk factors, statin use and baseline serum non-HDL cholesterol level, plasma Cer(d18:1/16:0) levels above vs below the median were significantly associated with MACE (HR: 2.63; 95% CI [1.44- 4.83], per ln(pmol/mL) p= 0.002) in ACS patients. In the full cohort and in SAP patients significant associations between plasma Cer(d18:1/16:0) levels above vs below the median and MACE could not be demonstrated (Figure 2).

Several lipid species displayed associations with the secondary endpoint (Figure 3 and Supplemental Table S1b); in univariable analysis, concentrations of Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:1), LacCer(d18:1/18:0), Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/20:0)/Cer(d18:1/24:0) and Cer(d18:1/24:1)/Cer(d18:1/24:0) were significantly associated with the composite endpoint of death or nonfatal ACS (Supplemental table S1b). After multivariable adjustment for cardiac risk factors, indication for CAG, statin use at baseline and serum LDL cholesterol level, except LacCer(d18:1/18:0) concentration, all of the above mentioned lipid species remained significantly associated with the composite endpoint of death or nonfatal ACS. Results remained materially the same after adjusting the multivariable models for serum non-HDL cholesterol level instead of serum LDL cholesterol level. The interaction terms between indication for CAG and Cer(d18:1/16:0) (p= 0.006), Cer(d18:1/24:1) (p= 0.025), Cer(d18:1/16:0)/Cer(d18:1/24:0) (p= 0.004) and Cer(d18:1/24:1)/Cer(d18:1/24:0) (p= 0.006) were significant on univariable and multivariable adjustment. All these associations were driven by the ACS patients (Supplemental table S3a and S3b). Interaction terms

between indication for CAG and the remaining molecular lipids and lipid ratios did not reach statistical significance.

DISCUSSION

We investigated the associations of ten previously identified high risk molecular lipid species and three ceramide ratios with clinical cardiovascular outcome during long-term follow-up in 581 patients. The main finding of our study was that higher Cer(d18:1/16:0) concentration and Cer(d18:1/24:1) concentration were significantly associated with MACE after multivariable adjustment for cardiac risk factors, clinical presentation and statin use at baseline. After additional adjustment for admission serum LDL cholesterol level or non-HDL cholesterol level, this association persisted only for Cer(d18:1/16:0) concentration. The latter association was driven by patients that presented with ACS. Another important finding of this study was that several lipid species were significantly and independently associated with the secondary endpoint, comprising the composite of all-cause mortality or nonfatal ACS. Likewise, several of these associations were driven by patients presenting with ACS, in whom we also observed higher plasma lipid concentrations as compared to patients with SAP.

Ceramides are a family of waxy lipid molecules and are composed of sphingosine and a fatty acid (17). They can be implicated in coronary artery disease through several mechanisms(3, 8). Ceramide is mainly produced through the SMase pathway, which breaks down sphingomyelin in the cell membrane and releases ceramides (17, 18). The production of ceramides can be increased by numerous cardiovascular risk factors such as oxidized-LDL and homocyteine (17). Moreover, inflammatory cytokines could also activate SMase and increase ceramide production mediated by increased reactive oxygen species (ROS) (17), which include HO_2, superoxide and hydroxyl radicals(17, 19). Ceramides can also act as signaling molecules regulating numerous cell responses and functions including the differentiation, proliferation, apoptosis and gene expression such as cytokines(17). Some of these roles of ceramides are associated with the molecular mechanisms of atherosclerosis and with plaque vulnerability (1, 17, 20).

The molecular lipids species in this study were chosen from the LURIC lipidomic study (1). The LURIC lipidomic study compared 258 male CAD patients who died within 3 years of follow-up with 187 matched control patients with CAD who did not die during the follow up. The chosen molecular lipid species were associated

with CAD outcome at the $p<0.05$ level. Our present results in essence validate the previous LURIC study, albeit with a somewhat different endpoint that also contains non-fatal adverse cardiovascular events. Several molecular lipid species such as CE 14:0, CE 18:3, CE 20:4, CE 20:5, CE 22:5, Cer(d18:1/24:0) were protective in the LURIC study but were not associated with clinical outcome in the present study. Conversely, Cer(d18:1/16:0), Cer(d18:1/20:0) and Cer(d18:1/24:1) were associated with mortality in the LURIC study and with clinical outcome in our present study. In a previous study ceramide long-chain-species were shown to mediate insulin resistance in mice and to be pro-apoptotic, whereas very-long-chain species were anti-apoptotic (21, 22). In our present study Cer(d18:1/16:0) and Cer(d18:1/20:0) were independently associated with clinical outcome and more harmful than Cer(d18:1/24:0). Interestingly Cer(d18:1/24:1) behaved contrarily when compared with Cer(d18:1/24:0). The reason for this difference remains to be investigated in further studies. Furthermore, in the LURIC study LacCer(d18:1/18:0) was associated with mortality, whereas in the current study there was only an association with clinical outcome (Supplemental Table S1B) in the unadjusted model. Based on previous observations LacCer (a glycosphingolipid) and other glucosylceramides appear to influence the atherogenic process in the atherosclerotic plaque by suppressing the production of macrophage apolipoprotein E leading to an accumulation of cholesterol in macrophage foam cells (19, 23). Moreover, in aortic smooth muscle cell LacCer is activated by oxidized-LDL and subsequently, LacCer enhances the activity of nicotinamide adenine dinucleotide phosphate oxidase to generate superoxide radicals which in turn mediate p44MAPK activation to enhance nuclear transcription factor expression and to stimulate the proliferation of smooth muscle cells, thereby contributing to atherosclerosis(2, 23). Altogether, as increased levels of LacCer mediate plaque formation, a relationship between LacCer and cardiovascular clinical outcome is to be expected. However in our study there was no independent association. Further studies are needed to establish the biological mechanisms of LacCer in CAD patients.

In our previous report on the current study population, Cer(d18:1/16:0) was an independent predictor of MACE during 1 year follow-up, and the three ceramide ratios were independent predictors of the composite endpoint of death or nonfatal ACS (20). Our current study confirms and extends the findings of our previous lipidomics analyses with shorter term follow-up (20).

Associations of lipids with incident acute MACE (all-cause mortality and nonfatal ACS) were more prominent than those with 'overall' MACE. Associations were also

more prominent in patients presenting with ACS than in those with SAP; although in the stratified analyses numbers of MACE were limited (90 in SAP patients and 65 in ACS patients), which may have influenced statistical significance, hazard ratios in SAP patients were also clearly closer to the null and interaction terms were significant. These findings are in line with pathophysiological insights from earlier studies. Patients with ACS have been shown to exhibit an increased pro-inflammatory and oxidative state compared to SAP patients (24, 25). This state even persists after stabilization (26, 27). High lipid plasma concentrations induce oxidative stress mediated by ROS (24, 28) and herewith further increase the risk of incident ACS. Furthermore, in ACS patients, so-called high-risk or vulnerable plaques have been shown to be more frequently present, which are more likely to lead to plaque rupture and thus to acute cardiac events (29, 30). These plaques have been shown to carry large lipid cores, and have been associated with high circulating lipid levels (20, 31).

In the last decade lipid species have received considerable attention as potential biomarkers in several lipid-related diseases (9). Furthermore, several molecular lipid species have been associated with the composition of atherosclerotic plaque (20) and with cardiovascular events during short-term follow-up (20, 32). However, clinical studies in patients with CAD on the association of lipid molecular species with cardiovascular outcome during long term follow-up are scarce. Laaksonen et al. (32) examined several ceramides and ceramide ratios in the Corogene cohort (80 stable CAD patients who died and 80 matched controls, 2.5 years follow-up), as well as in the BECAC cohort (1580 stable CAD patients, 81 of whom died during 4.6 years follow-up). In both cohorts Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1), Cer(d18:1/16:0)/Cer(d18:1/24:0), Cer(d18:1/18:0)/Cer(d18:1/24:0) and Cer(d18:1/24:1)/Cer(d18:1/24:0) were associated with cardiovascular death. Although Laaksonen et al. only examined cardiovascular death, and not (acute) MACE, the results with regard to the corresponding ceramides and ceramide ratios were in line with our results, even though the associations in our study were driven by patients presenting with ACS. Havulinna et al. (3), in the FINRISK cohort, measured 4 circulating ceramides in a healthy population, and within this population they examined a subgroup with prevalent or incident MACE. In these 396 patients, plasma Cer(d18:1/16:0) and Cer(d18:1)/24:1) concentrations were independent predictors of recurrent MACE (n=226) or fatal recurrent MACE (n=70) during a follow-up of 13 years. These results were in line with ours, although in our study the association between Cer(d18:1/24:1) and MACE was not independent of LDL or non-HDL cholesterol level. Conversely, its association with the secondary endpoint (nonfatal ACS or death) was independent

of LDL or non-HDL cholesterol level. Other studies on long-term prognostic value of lipidomics have mostly used healthy populations. In the Bruneck study, Stegemann et al.(33) analyzed the association of 135 lipid species (including CEs) with incident cardiovascular disease (CVD) during 10-year follow-up in a prospective population-based survey. They demonstrated significant associations for 28 lipids. Among these lipid species, 3 were most informative for CVD risk: TAG(54:2), CE(16:1) and PE(36:5). The results with regard to the CEs that were also measured in this study were in line with our results, i.e. no significant associations were found. Moreover, Alshehry et al. (2) examined the prognostic value of 310 plasma lipid species (including CEs and CERs) for cardiovascular risk stratification in a case-cohort of 3779 individuals with diabetes mellitus that included 698 patients with cardiovascular events and 355 patients with cardiovascular death. Multiple lipid species were significantly associated with cardiovascular events and cardiovascular death. In line with our study, a significant association was found between Cer(d18:1/24:1) and death. In contrast to our study, no association was found between plasma Cer(d18:1/16:0) and cardiovascular outcome. This may possibly be explained by differences in the study populations; in particular, the patients studied by Alshehry et al. were diagnosed with diabetes mellitus and had ≥ 1 additional cardiovascular risk factors. For the other lipid species, no significant associations were found.

Some limitations of this study need to be acknowledged. First, this study is an observational cohort study. Despite using multivariable analysis to adjust for possible confounders that may be related to the study outcomes, we cannot exclude the possibility of residual confounding. Second, a large proportion of the subjects were on lipid lowering medication, which will influence the plasma lipid concentrations in these individuals. However, all multivariable analyses were adjusted for statin use.

In conclusion, in patients with established CAD, plasma Cer(d18:1/16:0) was associated with MACE during a median follow-up time of 4.7 years, independently from established cardiac factors, statin use and serum LDL or non-HDL cholesterol level. Furthermore, after multivariable adjustment, concentrations of Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:1) and all ceramide ratios were associated with the secondary endpoint, comprising the composite of all-cause mortality or nonfatal ACS. Our results support the hypothesis that ceramide plasma concentrations and ratios predict long-term cardiovascular outcome and therefore circulating molecular lipids may further improve risk stratification of CAD patients.

REFERENCES

1. Tarasov, K., K. Ekroos, M. Suoniemi, D. Kauhanen, T. Sylvanne, R. Hurme, I. Gouni-Berthold, H. K. Berthold, M. E. Kleber, R. Laaksonen, and W. Marz. 2014. Molecular lipids identify cardiovascular risk and are efficiently lowered by simvastatin and PCSK9 deficiency. The Journal of clinical endocrinology and metabolism 99: E45-52.
2. Alshehry, Z. H., P. A. Mundra, C. K. Barlow, N. A. Mellett, G. Wong, M. J. McConville, J. Simes, A. M. Tonkin, D. R. Sullivan, E. H. Barnes, P. J. Nestel, B. A. Kingwell, M. Marre, B. Neal, N. R. Poulter, A. Rodgers, B. Williams, S. Zoungas, G. S. Hillis, J. Chalmers, M. Woodward, and P. J. Meikle. 2016. Plasma Lipidomic Profiles Improve on Traditional Risk Factors for the Prediction of Cardiovascular Events in Type 2 Diabetes Mellitus. Circulation 134: 1637-1650.
3. Havulinna, A. S., M. Sysi-Aho, M. Hilvo, D. Kauhanen, R. Hurme, K. Ekroos, V. Salomaa, and R. Laaksonen. 2016. Circulating Ceramides Predict Cardiovascular Outcomes in the Population-Based FINRISK 2002 Cohort. Arteriosclerosis, thrombosis, and vascular biology 36: 2424-2430.
4. Ekroos, K., M. Janis, K. Tarasov, R. Hurme, and R. Laaksonen. 2010. Lipidomics: a tool for studies of atherosclerosis. Current atherosclerosis reports 12: 273-281.
5. Watson, A. D. 2006. Thematic review series: systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: a global approach to lipid analysis in biological systems. J Lipid Res 47: 2101-2111.
6. Roberts, L. D., G. McCombie, C. M. Titman, and J. L. Griffin. 2008. A matter of fat: an introduction to lipidomic profiling methods. J Chromatogr B Analyt Technol Biomed Life Sci 871: 174-181.
7. Janis, M. T., R. Laaksonen, and M. Oresic. 2008. Metabolomic strategies to identify tissue- specific effects of cardiovascular drugs. Expert Opin Drug Metab Toxicol 4: 665-680.
8. Stock, J. 2012. The emerging role of lipidomics. Atherosclerosis 221: 38-40.
9. Hu, C., R. van der Heijden, M. Wang, J. van der Greef, T. Hankemeier, and G. Xu. 2009. Analytical strategies in lipidomics and applications in disease biomarker discovery. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences 877: 2836-2846.
10. de Boer, S. P., J. M. Cheng, H. M. Garcia-Garcia, R. M. Oemrawsingh, R. J. van Geuns, E. Regar, F. Zijlstra, R. Laaksonen, E. Halperin, M. E. Kleber, W. Koenig, E. Boersma, and P. W. Serruys. 2014. 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 10: 953-960.
11. Heiskanen, L. A., M. Suoniemi, H. X. Ta, K. Tarasov, and K. Ekroos. 2013. Long-term performance and stability of molecular shotgun lipidomic analysis of human plasma samples. Anal Chem 85: 8757-8763.
12. Merrill, A. H., Jr., M. C. Sullards, J. C. Allegood, S. Kelly, and E. Wang. 2005. Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. Methods 36: 207-224.
13. Ejsing, C. S., E. Duchoslav, J. Sampaio, K. Simons, R. Bonner, C. Thiele, K. Ekroos, and A. Shevchenko. 2006. Automated identification and quantification of glycerophospholipid molecular species by multiple precursor ion scanning. Anal Chem 78: 6202-6214.

14. Roffi, M., C. Patrono, J. P. Collet, C. Mueller, M. Valgimigli, F. Andreotti, J. J. Bax, M. A. Borger, C. Brotons, D. P. Chew, B. Gencer, G. Hasenfuss, K. Kjeldsen, P. Lancellotti, U. Landmesser, J. Mehilli, D. Mukherjee, R. F. Storey, S. Windecker, H. Baumgartner, O. Gaemperli, S. Achenbach, S. Agewall, L. Badimon, C. Baigent, H. Bueno, R. Bugiardini, S. Carerj, F. Casselman, T. Cuisset, C. Erol, D. Fitzsimons, M. Halle, C. Hamm, D. Hildick-Smith, K. Huber, E. Iliodromitis, S. James, B. S. Lewis, G. Y. Lip, M. F. Piepoli, D. Richter, T. Rosemann, U. Sechtem, P. G. Steg, C. Vrints, J. Luis Zamorano, and S. T. S. E. o. t. E. S. o. C. Management of Acute Coronary Syndromes in Patients Presenting without Persistent. 2016. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). Eur Heart J 37: 267-315.
15. Ibanez, B., S. James, S. Agewall, M. J. Antunes, C. Bucciarelli-Ducci, H. Bueno, A. L. P. Caforio, F. Crea, J. A. Goudevenos, S. Halvorsen, G. Hindricks, A. Kastrati, M. J. Lenzen, E. Prescott, M. Roffi, M. Valgimigli, C. Varenhorst, P. Vranckx, and P. Widimsky. 2017. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). European heart journal.
16. Stone, G. W., A. Maehara, A. J. Lansky, B. de Bruyne, E. Cristea, G. S. Mintz, R. Mehran, J. McPherson, N. Farhat, S. P. Marso, H. Parise, B. Templin, R. White, Z. Zhang, P. W. Serruys, and P. Investigators. 2011. A prospective natural-history study of coronary atherosclerosis. N Engl J Med 364: 226-235.
17. Bismuth, J., P. Lin, Q. Yao, and C. Chen. 2008. Ceramide: a common pathway for atherosclerosis? Atherosclerosis 196: 497-504.
18. Merrill, A. H., Jr., E. M. Schmelz, D. L. Dillehay, S. Spiegel, J. A. Shayman, J. J. Schroeder, R. T. Riley, K. A. Voss, and E. Wang. 1997. Sphingolipids--the enigmatic lipid class: biochemistry, physiology, and pathophysiology. Toxicology and applied pharmacology 142: 208-225.
19. Garner, B., H. R. Mellor, T. D. Butters, R. A. Dwek, and F. M. Platt. 2002. Modulation of THP-1 Macrophage and Cholesterol-Loaded Foam Cell Apolipoprotein E Levels by Glycosphingolipids. Biochemical and Biophysical Research Communications 290: 1361-1367.
20. Cheng, J. M., M. Suoniemi, I. Kardys, T. Vihervaara, S. P. de Boer, K. M. Akkerhuis, M. Sysi-Aho, K. Ekroos, H. M. Garcia-Garcia, R. M. Oemrawsingh, E. Regar, W. Koenig, P. W. Serruys, R. J. van Geuns, E. Boersma, and R. Laaksonen. 2015. Plasma concentrations of molecular lipid species in relation to coronary plaque characteristics and cardiovascular outcome: Results of the ATHEROREMO-IVUS study. Atherosclerosis 243: 560-566.
21. Turpin, S. M., H. T. Nicholls, D. M. Willmes, A. Mourier, S. Brodesser, C. M. Wunderlich, J. Mauer, E. Xu, P. Hammerschmidt, H. S. Bronneke, A. Trifunovic, G. LoSasso, F. T. Wunderlich, J. W. Kornfeld, M. Bluher, M. Kronke, and J. C. Bruning. 2014. Obesity-induced CerS6-dependent C16:0 ceramide production promotes weight gain and glucose intolerance. Cell metabolism 20: 678-686.

22. Raichur, S., S. T. Wang, P. W. Chan, Y. Li, J. Ching, B. Chaurasia, S. Dogra, M. K. Ohman, K. Takeda, S. Sugii, Y. Pewzner-Jung, A. H. Futerman, and S. A. Summers. 2014. CerS2 haploinsufficiency inhibits beta-oxidation and confers susceptibility to diet-induced steatohepatitis and insulin resistance. Cell metabolism 20: 687-695.
23. Chatterjee, S. B., S. Dey, W. Y. Shi, K. Thomas, and G. M. Hutchins. 1997. Accumulation of glycosphingolipids in human atherosclerotic plaque and unaffected aorta tissues. Glycobiology 7: 57-65.
24. Libby, P., P. M. Ridker, and A. Maseri. 2002. Inflammation and atherosclerosis. Circulation 105: 1135-1143.
25. Lorgis, L., M. Zeller, G. Dentan, P. Sicard, C. Richard, P. Buffet, I. L'Huillier, J. C. Beer, Y. Cottin, L. Rochette, and C. Vergely. 2010. The free oxygen radicals test (FORT) to assess circulating oxidative stress in patients with acute myocardial infarction. Atherosclerosis 213: 616-621.
26. Ridker, P. M., N. Rifai, M. Pfeffer, F. Sacks, S. Lepage, and E. Braunwald. 2000. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. Circulation 101: 2149-2153.
27. Patel, P. J., A. V. Khera, K. Jafri, R. L. Wilensky, and D. J. Rader. 2011. 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 58: 2068-2075.
28. Witztum, J. L. 1994. The oxidation hypothesis of atherosclerosis. Lancet (London, England) 344: 793-795.
29. Hong, M. K., G. S. Mintz, C. W. Lee, Y. H. Kim, S. W. Lee, J. M. Song, K. H. Han, D. H. Kang, J. K. Song, J. J. Kim, S. W. Park, and S. J. Park. 2004. Comparison of coronary plaque rupture between stable angina and acute myocardial infarction: a three-vessel intravascular ultrasound study in 235 patients. Circulation 110: 928-933.
30. Goldstein, J. A., D. Demetriou, C. L. Grines, M. Pica, M. Shoukfeh, and W. W. O'Neill. 2000. Multiple complex coronary plaques in patients with acute myocardial infarction. N Engl J Med 343: 915-922.
31. Falk, E., P. K. Shah, and V. Fuster. 1995. Coronary plaque disruption. Circulation 92: 657-671.
32. Laaksonen, R., K. Ekroos, M. Sysi-Aho, M. Hilvo, T. Vihervaara, D. Kauhanen, M. Suoniemi, R. Hurme, W. Marz, H. Scharnagl, T. Stojakovic, E. Vlachopoulou, M. L. Lokki, M. S. Nieminen, R. Klingenberg, C. M. Matter, T. Hornemann, P. Juni, N. Rodondi, L. Raber, S. Windecker, B. Gencer, E. R. Pedersen, G. S. Tell, O. Nygard, F. Mach, J. Sinisalo, and T. F. Luscher. 2016. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. Eur Heart J 37: 1967-1976.
33. Stegemann, C., R. Pechlaner, P. Willeit, S. R. Langley, M. Mangino, U. Mayr, C. Menni, A. Moayyeri, P. Santer, G. Rungger, T. D. Spector, J. Willeit, S. Kiechl, and M. Mayr. 2014. Lipidomics profiling and risk of cardiovascular disease in the prospective population-based Bruneck study. Circulation 129: 1821-1831.

SUPPLEMENTARY DATA

Supplemental Table S1. Association between molecular lipids concentrations and cardiovascular outcome in the full cohort.

Supplemental Table S1a. Association with MACE in the full cohort (n=574)	Unadjusted		Adjusted for cardiac risk factors* + Statin use †		Adjusted for cardiac risk factors* + Statin use † + serum LDL cholesterol ‡		Adjusted for cardiac risk factors* + Statin use † + serum non-HDL cholesterol§	
	HR (95%CI)	P-value	HR (95%CI)	P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
CE 14:0	0.95 (0.67-1.35)	0.768	1.12 (0.77-1.64)	0.542	0.94 (0.59-1.50)	0.786	0.90 (0.56-1.46)	0.697
CE 18:3	0.91 (0.63-1.33)	0.640	1.27 (0.83-1.93)	0.267	1.10 (0.65-1.86)	0.734	1.01 (0.63-1.83)	0.799
CE 20:4	0.70 (0.41-1.20)	0.199	0.80 (0.46-1.42)	0.451	0.63 (0.34-1.18)	0.150	0.64 (0.35-1.18)	0.153
CE 20:5	1.11 (0.83-1.49)	0.483	1.11 (0.82-1.47)	0.543	1.01 (0.73-1.40)	0.950	1.01 (0.73-1.39)	0.954
CE 22:5	0.98 (0.73-1.33)	0.914	1.16 (0.86-1.57)	0.327	1.10 (0.79-1.52)	0.584	1.09 (0.79-1.51)	0.595
Cer (d18:1/16:0)	1.40 (0.83-2.37)	0.206	2.14 (1.22-3.76)	0.008	2.16 (1.09-4.27)	0.027	2.32 (1.09-4.96)	0.030
Cer (d18:1/20:0)	1.23 (0.79-1.92)	0.363	1.52 (0.94-2.46)	0.085	1.40 (0.83-2.35)	0.206	1.37 (0.78-2.40)	0.280
Cer (d18:1/24:0)	0.97 (0.62-1.52)	0.882	1.33 (0.81-2.19)	0.258	1.12 (0.61-2.08)	0.716	1.01 (0.55-2.07)	0.858
Cer (d18:1/24:1)	1.32 (0.84-2.09)	0.230	1.64 (1.00-2.68)	0.049	1.52 (0.86-2.70)	0.151	1.53 (0.81-2.90)	0.153
LacCer (d18:1/18:0)	1.11 (0.69-1.79)	0.675	1.23 (0.75-2.03)	0.418	1.05 (0.60-1.85)	0.860	1.05 (0.61-1.83)	0.858
Cer (d18:1/16:0)/ Cer(d18:1/24:0)	1.79 (0.95-3.37)	0.070	1.82 (0.90-3.68)	0.096	1.95 (0.96-3.96)	0.064	1.94 (0.96-3.91)	0.065
Cer (d18:1/20:0)/ Cer(d18:1/24:0)	1.38 (0.82-2.32)	0.220	1.24 (0.70-2.17)	0.462	1.37 (0.77-2.46)	0.289	1.31 (0.74-2.33)	0.348
Cer (d18:1/24:1)/ Cer(d18:1/24:0)	1.80 (0.96-3.38)	0.067	1.52 (0.76-3.03)	0.234	1.59 (0.79-3.21)	0.194	1.54 (0.77-3.10)	0.223

Supplemental Table S1b. Association with ACS or death in the full cohort (n=574)	Unadjusted		Adjusted for cardiac risk factors + Statin use		Adjusted for cardiac risk factors + Statin use + serum LDL cholesterol		Adjusted for cardiac risk factors* + Statin use + serum non-HDL cholesterol§	
	HR (95%CI)	P-value		P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
CE 14:0	0.93 (0.60-1.44)	0.728	1.17 (0.67-2.04)	0.587	0.91 (0.50-1.67)	0.765	0.82 (0.44-1.53)	0.535
CE 18:3	0.77 (0.48-1.22)	0.263	1.00 (0.60-1.67)	0.993	0.86 (0.44-1.68)	0.666	0.79 (0.40-1.53)	0.479
CE 20:4	0.76 (0.39-1.49)	0.426	0.89 (0.43-1.84)	0.757	0.80 (0.36-1.75)	0.572	0.76 (0.35-1.65)	0.489
CE 20:5	1.06 (0.74-1.51)	0.760	1.02 (0.71-1.48)	0.904	0.98 (0.66-1.46)	0.982	0.96 (0.65-1.43)	0.836
CE 22:5	0.85 (0.58-1.24)	0.400	0.94 (0.64-1.37)	0.735	0.89 (0.59-1.35)	0.581	0.87 (0.58-1.31)	0.504
Cer (d18:1/16:0)	2.03 (1.07-3.86)	0.032	2.37 (1.17-4.79)	0.017	2.98 (1.28-6.93)	0.011	3.19 (1.24-8.20)	0.016
Cer (d18:1/20:0)	1.92 (1.11-3.34)	0.020	2.05 (1.12-3.75)	0.020	2.12 (1.11-4.06)	0.023	2.20 (1.08-4.45)	0.030
Cer (d18:1/24:0)	0.87 (0.50-1.50)	0.611	1.10 (0.61-1.99)	0.759	0.98 (0.46-2.07)	0.950	0.85 (0.38-1.89)	0.683
Cer (d18:1/24:1)	1.93 (1.10-3.38)	0.022	2.00 (1.01-3.61)	0.027	2.23 (1.11-4.50)	0.025	2.35 (1.07-5.20)	0.034
LacCer (d18:1/18:0)	1.90 (1.05-3.42)	0.033	1.71 (0.92-3.18)	0.091	1.76 (0.88-3.51)	0.109	1.66 (0.83-3.29)	0.150
Cer (d18:1/16:0)/ Cer(d18:1/24:0)	3.67 (1.77-7.62)	<0.001	2.90 (1.25-6.71)	0.013	3.09 (1.32-7.20)	0.009	3.13 (1.35-7.25)	0.008
Cer (d18:1/20:0)/ Cer(d18:1/24:0)	2.93 (1.54-5.57)	0.001	2.24 (1.12-4.49)	0.022	2.54 (1.23-5.26)	0.012	2.48 (1.22-5.06)	0.012
Cer (d18:1/24:1)/ Cer(d18:1/24:0)	4.65 (2.12-10.2)	<0.001	3.10 (1.31-7.33)	0.010	3.26 (1.36-7.84)	0.008	3.22 (1.35-7.71)	0.009

The results are presented as hazards ratios (HR) with 95 % confidence intervals (CI) per unit increase in Ln-transformed lipid concentration.
ACS, acute coronary syndrome; CE, cholesteryl ester; Cer, ceramide; HR, hazard ratio; LacCer, lactosylceramide; LDL, low-density lipoprotein; MACE, major adverse cardiac events.

* Cardiac risk factors include: age, gender, diabetes mellitus, hypertension, hypercholesterolemia, and clinical presentation (ACS or SAP).
† Statin use was registered at the time of hospital admission.
‡ Serum LDL concentration was measured prior to coronary angiography.
§ Serum non-HDL concentration was measured prior to coronary angiography and is calculated as: Total cholesterol concentration minus the HDL cholesterol concentration.

Supplemental Table S2. Association between molecular lipids concentrations and MACE in patients with ACS or SAP.

Supplemental Table S2a. Association with MACE in ACS patients (n=313)	Unadjusted		Adjusted for cardiac risk factors + Statin use		Adjusted for cardiac risk factors + Statin use + serum LDL cholesterol		Adjusted for cardiac risk factors* + Statin use + serum non-HDL cholesterol§	
	HR (95%CI)	P-value		P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
CE 14:0	0.82 (0.48-1.42)	0.485	0.92 (0.51-1.65)	0.776	0.68 (0.31-1.49)	0.332	0.70 (0.31-1.55)	0.376
CE 18:3	0.94 (0.53-1.67)	0.843	1.34 (0.71-2.54)	0.368	1.29 (0.58-2.89)	0.533	1.35 (0.61-3.02)	0.460
CE 20:4	0.56 (0.24-1.32)	0.186	0.62 (0.24-1.60)	0.321	0.50 (0.18-1.38)	0.177	0.51 (0.18-1.41)	0.195
CE 20:5	1.15 (0.73-1.80)	0.543	1.13 (0.71-1.80)	0.605	1.07 (0.64-1.80)	0.793	1.09 (0.66-1.82)	0.732
CE 22:5	0.91 (0.54-1.52)	0.709	1.03 (0.61-1.76)	0.908	0.97 (0.54-1.72)	0.906	0.98 (0.55-1.75)	0.945
Cer (d18:1/16:0)	2.75 (1.12-6.75)	0.027	2.96 (1.16-7.60)	0.024	4.16 (1.31-13.2)	0.015	6.13 (1.65-22.8)	0.007
Cer (d18:1/20:0)	1.45 (0.72-2.93)	0.298	1.52 (0.72-3.22)	0.271	1.46 (0.65-3.32)	0.360	1.54 (0.65-3.66)	0.330
Cer (d18:1/24:0)	1.01 (0.52-1.98)	0.970	1.38 (0.66-2.89)	0.396	1.32 (0.50-3.44)	0.574	1.44 (0.51-4.05)	0.495
Cer (d18:1/24:1)	1.79 (0.88-3.63)	0.107	1.81 (0.86-3.85)	0.120	1.99 (0.80-4.96)	0.141	2.32 (0.85-6.33)	0.100
LacCer (d18:1/18:0)	1.19 (0.57-2.51)	0.644	1.11 (0.51-2.39)	0.799	0.99 (0.42-2.33)	0.974	1.02 (0.44-2.39)	0.963
Cer (d18:1/16:0)/ Cer(d18:1/24:0)	3.01 (1.27-7.44)	0.013	2.25 (0.77-6.55)	0.137	2.51 (0.84-7.56)	0.101	2.45 (0.82-7.33)	0.109
Cer (d18:1/20:0)/ Cer(d18:1/24:0)	1.51 (0.71-3.20)	0.280	1.11 (0.49-2.51)	0.795	1.22 (0.52-2.85)	0.651	1.18 (0.51-2.72)	0.702
Cer (d18:1/24:1)/ Cer(d18:1/24:0)	2.71 (1.06-6.94)	0.038	1.70 (0.58-4.96)	0.332	1.78 (0.60-5.26)	0.299	1.74 (0.59-5.12)	0.312

Supplemental Table S2b. Association with MACE in SAP patients (n=261)	Unadjusted		Adjusted for cardiac risk factors + Statin use		Adjusted for cardiac risk factors + Statin use + serum LDL cholesterol		Adjusted for cardiac risk factors* + Statin use + serum non-HDL cholesterol§	
	HR (95%CI)	P-value		P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
CE 14:0	1.22 (0.75-2.00)	0.424	1.37 (0.82-2.28)	0.230	1.18 (0.65-2.15)	0.591	1.13 (0.61-2.10)	0.702
CE 18:3	1.06 (0.63-1.78)	0.819	1.25 (0.71-2.20)	0.432	0.96 (0.47-1.97)	0.907	0.91 (0.44-1.89)	0.808
CE 20:4	0.89 (0.45-1.76)	0.732	0.93 (0.46-1.91)	0.848	0.70 (0.32-1.53)	0.367	0.72 (0.33-1.55)	0.394
CE 20:5	1.10 (0.75-1.61)	0.641	1.08 (0.72-1.61)	0.713	0.97 (0.63-1.48)	0.870	0.96 (0.63-1.47)	0.861
CE 22:5	1.11 (0.78-1.58)	0.574	1.30 (0.89-1.89)	0.181	1.20 (0.80-1.82)	0.381	1.20 (0.80-1.81)	0.384
Cer (d18:1/16:0)	1.61 (0.79-3.26)	0.188	1.66 (0.80-3.45)	0.172	1.34 (0.55-3.23)	0.521	1.22 (0.45-3.29)	0.698
Cer (d18:1/20:0)	1.48 (0.81-2.72)	0.205	1.45 (0.77-2.74)	0.253	1.25 (0.63-2.49)	0.520	1.13 (0.62-2.42)	0.761
Cer (d18:1/24:0)	1.27 (0.66-2.47)	0.474	1.33 (0.68-2.59)	0.410	1.00 (0.44-2.26)	1.000	0.88 (0.36-2.14)	0.777
Cer (d18:1/24:1)	1.42 (0.75-2.66)	0.281	1.44 (0.75-2.78)	0.278	1.19 (0.57-2.49)	0.652	1.05 (0.46-2.42)	0.901
LacCer (d18:1/18:0)	1.33 (0.702.52)	0.383	1.27 (0.65-2.46)	0.488	1.01 (0.48-2.14)	0.986	1.02 (0.49-2.10)	0.966
Cer (d18:1/16:0)/ Cer(d18:1/24:0)	1.37 (0.55-3.42)	0.504	1.32 (0.50-3.51)	0.578	1.40 (0.54-3.65)	0.488	1.39 (0.54-3.60)	0.493
Cer (d18:1/20:0)/ Cer(d18:1/24:0)	1.33 (0.63-2.81)	0.455	1.19 (0.54-2.62)	0.661	1.37 (0.61-3.01)	0.451	1.26 (0.57-2.78)	0.571
Cer (d18:1/24:1)/ Cer(d18:1/24:0)	1.27 (0.54-2.98)	0.588	1.20 (0.48-3.00)	0.700	1.31 (0.51-3.33)	0.575	1.23 (0.49-3.10)	0.668

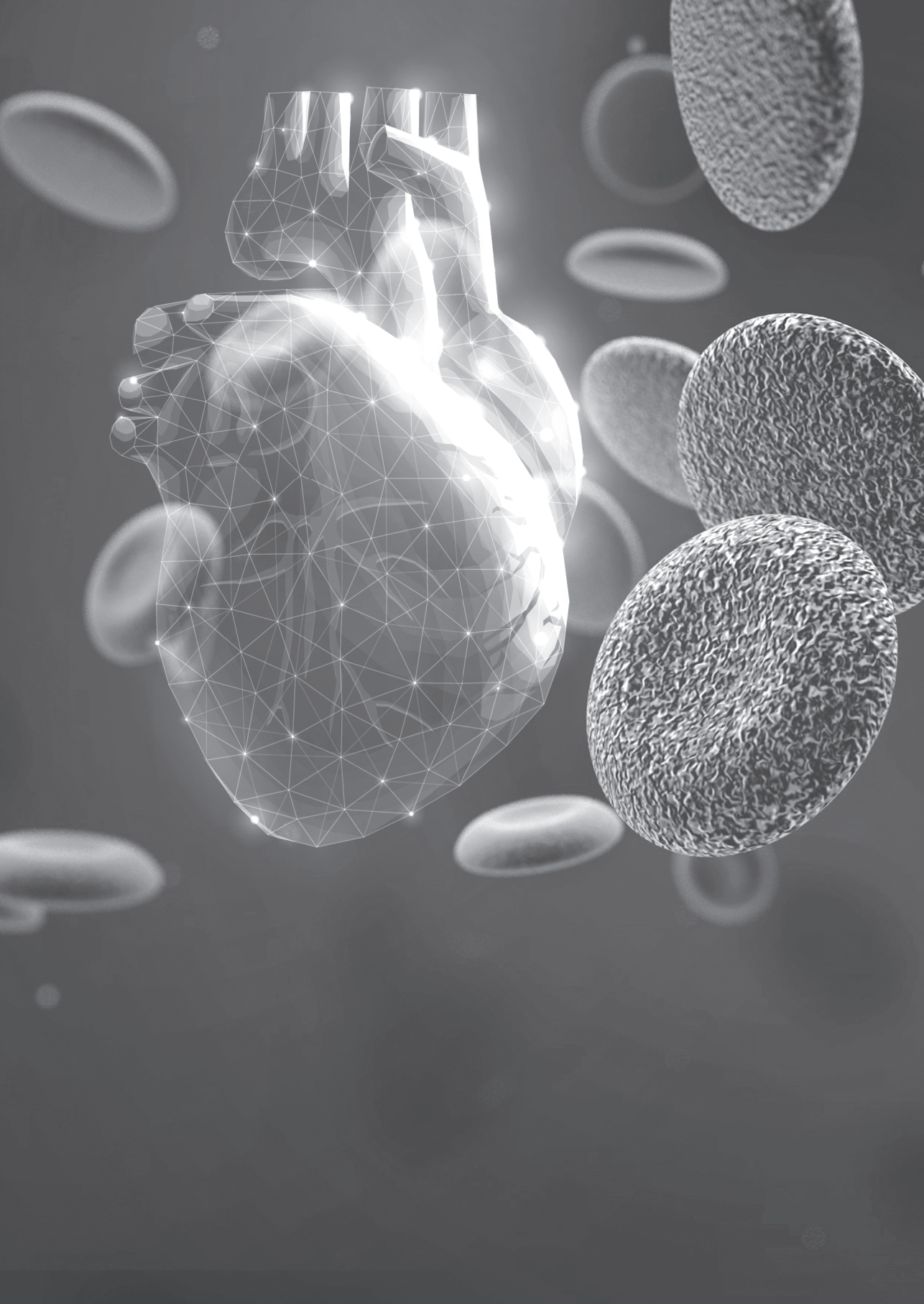
The results are presented as hazards ratios (HR) with 95 % confidence intervals (CI) per unit increase in Ln-transformed lipid concentration.
ACS, acute coronary syndrome; CE, cholesteryl ester; Cer, ceramide; HR, hazard ratio; LacCer, lactosylceramide; LDL, low-density lipoprotein; SAP, stable angina pectoris.

Supplemental Table S3. Association between molecular lipids concentrations and death or nonfatal ACS in patients with ACS or SAP.

Supplemental Table S3a. Association with death or ACS in ACS patients (n=313)	Unadjusted		Adjusted for cardiac risk factors + Statin use		Adjusted for cardiac risk factors + Statin use + serum LDL cholesterol		Adjusted for cardiac risk factors* + Statin use + serum non-HDL cholesterol§	
	HR (95%CI)	P-value		P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
CE 14:0	0.78 (0.42-1.45)	0.426	0.84 (0.43-1.64)	0.613	0.57 (0.23-1.44)	0.237	0.53 (0.21-1.35)	0.182
CE 18:3	0.73 (0.38-1.38)	0.330	0.97 (0.48-1.96)	0.931	0.77 (0.31-1.94)	0.577	0.74 (0.30-1.86)	0.525
CE 20:4	0.60 (0.23-1.59)	0.305	0.63 (0.21-1.92)	0.420	0.51 (0.15-1.68)	0.267	0.49 (0.15-1.62)	0.243
CE 20:5	1.21 (0.73-2.02)	0.465	1.16 (0.68-1.97)	0.594	1.11 (0.62-2.00)	0.732	1.10 (0.61-1.97)	0.758
CE 22:5	0.78 (0.44-1.41)	0.416	0.89 (0.48-1.67)	0.725	0.81 (0.41-1.61)	0.547	0.80 (0.40-1.58)	0.514
Cer (d18:1/16:0)	4.08 (1.47-11.3)	0.007	4.16 (1.41-12.29)	0.010	7.41 (2.01-27.40)	0.003	11.14 (2.44-50.8)	0.002
Cer (d18:1/20:0)	1.93 (0.87-4.27)	0.107	1.85 (0.79-4.38)	0.159	1.86 (0.73-4.72)	0.191	1.88 (0.70-5.01)	0.210
Cer (d18:1/24:0)	0.84 (0.40-1.77)	0.646	1.12 (0.50-2.53)	0.788	0.94 (0.32-2.79)	0.908	0.86 (0.28-2.67)	0.797
Cer (d18:1/24:1)	2.61 (1.18-5.78)	0.018	2.48 (1.06-5.78)	0.036	3.21 (1.17-8.82)	0.024	3.67 (1.20-11.25)	0.023
LacCer (d18:1/18:0)	1.70 (0.74-3.90)	0.213	1.50 (0.64-3.54)	0.354	1.46 (0.60-3.82)	0.440	1.42 (0.55-3.68)	0.471
Cer (d18:1/16:0)/ Cer(d18:1/24:0)	6.18 (2.44-15.6)	<0.001	5.08 (1.57-16.41)	0.007	5.91 (1.76-19.89)	0.004	5.88 (1.76-19.72)	0.004
Cer (d18:1/20:0)/ Cer(d18:1/24:0)	2.65 (1.14-6.17)	0.024	1.76 (0.70-4.44)	0.228	2.03 (0.77-5.41)	0.155	1.99 (0.76-5.23)	0.160
Cer (d18:1/24:1)/ Cer(d18:1/24:0)	8.06 (2.77-23.5)	<0.001	5.11 (1.47-17.75)	0.010	5.57 (1.55-19.97)	0.008	5.43 (1.53-19.23)	0.009

Supplemental Table 3b. Association with death or ACS in SAP patients (n=261)	Unadjusted		Adjusted for cardiac risk factors + Statin use		Adjusted for cardiac risk factors + Statin use + serum LDL cholesterol		Adjusted for cardiac risk factors* + Statin use + serum non-HDL cholesterol§	
	HR (95%CI)	P-value		P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
CE 14:0	1.18 (0.61-2.28)	0.624	1.37 (0.68-2.75)	0.378	1.41 (0.61-3.28)	0.425	1.26 (0.53-2.98)	0.595
CE 18:3	0.87 (0.44-1.75)	0.702	1.08 (0.50-2.32)	0.841	0.96 (0.35-2.59)	0.931	0.82 (0.30-2.23)	0.695
CE 20:4	0.97 (0.38-2.45)	0.945	1.18 (0.45-3.07)	0.739	1.10 (0.38-3.19)	0.856	1.03 (0.37-2.87)	0.962
CE 20:5	0.92 (0.56-1.53)	0.754	0.88 (0.51-1.49)	0.624	0.83 (0.47-1.45)	0.510	0.80 (0.46-1.41)	0.446
CE 22:5	0.95 (0.58-1.54)	0.827	1.04 (0.64-1.71)	0.875	1.00 (0.58-1.73)	0.998	0.97 (0.56-1.66)	0.901
Cer (d18:1/16:0)	1.46 (0.57-3.76)	0.429	1.45 (0.53-4.00)	0.471	1.41 (0.42-4.71)	0.577	1.19 (0.31-4.50)	0.801
Cer (d18:1/20:0)	2.22 (1.00-4.96)	0.051	2.25 (0.93-5.44)	0.071	2.33 (0.91-5.99)	0.079	2.39 (0.83-6.88)	0.108
Cer (d18:1/24:0)	0.99 (0.42-2.32)	0.981	1.12 (0.46-2.70)	0.803	0.99 (0.34-2.92)	0.989	0.79 (0.24-2.60)	0.704
Cer (d18:1/24:1)	1.58 (0.69-3.63)	0.281	1.47 (0.61-3.58)	0.394	1.44 (0.53-3.88)	0.476	1.29 (0.42-3.93)	0.660
LacCer (d18:1/18:0)	2.38 (1.01-5.61)	0.047	2.02 (0.80-5.09)	0.135	2.13 (0.76-5.97)	0.151	1.92 (0.70-5.32)	0.208
Cer (d18:1/16:0)/ Cer(d18:1/24:0)	1.85 (0.57-5.97)	0.305	1.43 (0.39-5.27)	0.587	1.51 (0.41-5.54)	0.538	1.56 (0.43-5.66)	0.499
Cer (d18:1/20:0)/ Cer(d18:1/24:0)	3.44 (1.25-9.45)	0.016	2.73 (0.94-7.94)	0.064	3.14 (1.04-9.53)	0.043	3.01 (1.02-8.87)	0.046
Cer (d18:1/24:1)/ Cer(d18:1/24:0)	2.34 (0.75-7.33)	0.143	1.64 (0.49-5.49)	0.424	1.73 (0.50-5.96)	0.384	1.71 (0.50-5.84)	0.391

The results are presented as hazards ratios (HR) with 95 % confidence intervals (CI) per unit increase in Ln-transformed lipid concentration.
ACS, acute coronary syndrome; CE, cholesteryl ester; Cer, ceramide; HR, hazard ratio; LacCer, lactosylceramide; LDL, low-density lipoprotein; SAP, stable angina pectoris.



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Association of serum PCSK9 with NIRS-derived lipid core burden index and long-term cardiac outcome (ATHEROREMO-NIRS substudy)

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Association of serum PCSK9 with NIRS-derived lipid core burden index and long-term cardiac outcome (ATHEROREMO-NIRS substudy)

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Submitted

ABSTRACT

Aims: We investigated the association between serum proprotein convertase subtilisin/kexin type 9 (PCSK9) levels, and near-infrared spectroscopy (NIRS)-derived lipid core burden index (LCBI) and the occurrence of major adverse cardiac events (MACE) in patients with coronary artery disease (CAD).

Methods: Serum PCSK9 levels were measured in 576 CAD patients who underwent diagnostic coronary angiography (CAG). NIRS imaging was performed in a subset of 203 patients, in a non-culprit coronary artery segment. Data on all-cause mortality, nonfatal ACS or unplanned coronary revascularization was collected during a median follow-up of 4.7 years.

Results: In multivariable analysis, serum PCSK9 was positively associated with LCBI (mean increase of 0.390 (95% CI [0.011-0.769] ZlnLCBI per unit increase in lnPCSK9 level p =0.044)). During a median follow-up of 4.7 years, 155 patients (27%) had MACE. After multivariable adjustment, serum PCSK9 levels showed a tendency towards an association with MACE (HR [95%CI]: 1.64[0.99-2.71], p=0.055) and a positive association with the composite of death or ACS (HR [95%CI]: 1.88[1.01-3.51], p=0.047). Patients admitted with serum PCSK9 levels above the median of 270 μg/L had 53% higher risk of MACE and 67% higher risk of death or ACS than those with levels below the median.

Conclusion: CAD patients with elevated serum PCSK9 levels had a higher NIRS-derived LCBI and higher incidence of adverse cardiac outcome than those with lower levels.

INTRODUCTION

The proprotein convertase subtilisin/kexin type 9 (PCSK9) enzyme plays a central role in the regulation of cholesterol homeostasis by increasing the endosomal and lysosomal degradation of hepatic low-density lipoprotein (LDL) receptors, resulting in increased serum LDL cholesterol (LDL-C) concentrations (1). In the last decade, PCSK9 enzyme has received substantial attention. Genetic studies have shown that loss-of-function mutations in the PCSK9 gene are associated with hypocholesterolemia and a decreased cardiovascular risk (2, 3). Recently, large phase 2 and phase 3 clinical trials have shown that PCSK9 inhibitors effectively reduce LDL-C levels, decrease plaque burden as assessed by intravascular ultrasound (IVUS) and reduce the risk of cardiovascular events (4-8). Moreover, in a previous study, we have shown that the serum PCSK9 level displayed a positive association with the amount of necrotic core tissue in coronary atherosclerotic plaque as assessed by IVUS, as well as with adverse cardiovascular outcome during 1 year follow-up, independent of serum LDL-C levels and statin use (9).

The catheter based near-infrared spectroscopy system (NIRS) is an intracoronary imaging technique capable of identifying lipid rich core-containing plaques in the coronary artery wall (10). Lipid rich core-containing plaques have been shown to be more vulnerable to rupture than plaques without a lipid rich core (11). Furthermore, in previous studies, we have demonstrated a strong association between a high NIRS-derived lipid core burden index (LCBI) and adverse cardiovascular events during short-term, as well as long-term follow-up (12, 13). However, there are currently no data on the association between serum PCSK9 levels and NIRS-derived LCBI.

Therefore, the primary aim of the current study is to investigate the relationship between serum PCSK9 levels and NIRS-derived LCBI in patients with coronary artery disease (CAD) undergoing coronary angiography (CAG). Secondly, we investigated whether the association between serum PCSK9 levels and the occurrence of major adverse cardiac events (MACE) persists during longer-term follow-up (9).

METHODS

Study population and design

The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis–intravascular Ultrasound (ATHEROREMO-IVUS) study, and its NIRS sub-study have been described in detail elsewhere (12-14). In brief, 768

patients were included in the ATHEROREMO-IVUS study between 2008 and 2011 at Erasmus MC, Rotterdam, the Netherlands. All patients included were 18 years or older and had an indication for CAG or percutaneous coronary intervention (PCI) due to an acute coronary syndrome (ACS) or stable angina pectoris (SAP). The flow chart of the study is shown in Figure 1. After the initial procedure, NIRS of a non-culprit vessel was performed in a subset of 203 patients. The medical ethics committee of Erasmus MC approved the ATHEROREMO-IVUS study and its NIRS sub-study and written informed consent was obtained from all patients. The ATHEROREMO-IVUS study and its NIRS sub-study were performed in accordance with the declaration of Helsinki.

Serum PCSK9 levels

Prior to the CAG or PCI, blood samples were collected from the arterial sheath and transported to the clinical laboratory of Erasmus MC within 2 hours for storage at a temperature of −80 °C. Serum samples were available for PCSK9 measurements

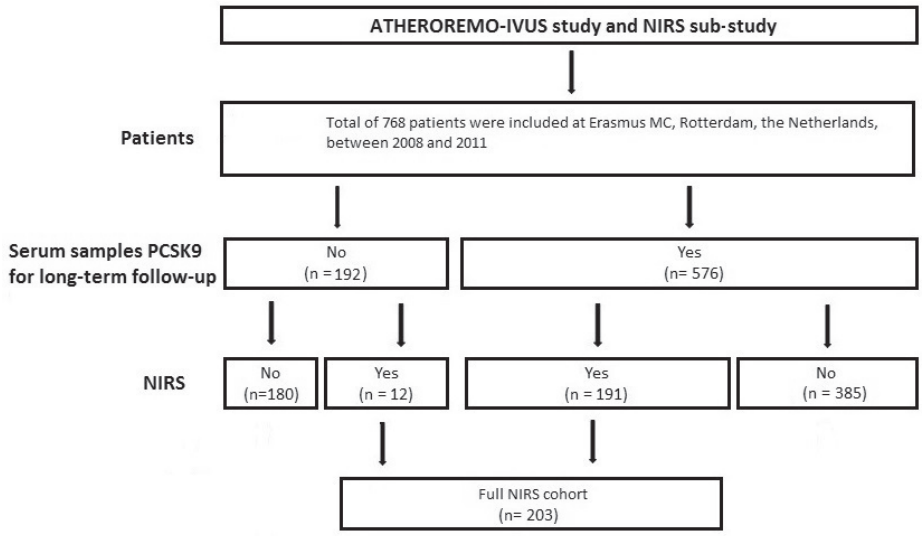


Figure 1. Flow chart ATHEROREMO-IVUS study and NIRS sub-study
Serum samples PCSK9 were measured in 576 CAD patients who underwent diagnostic coronary angiography for stable angina pectoris or acute coronary syndrome. NIRS imaging was performed in a subset of 203 patients, in a non-culprit coronary artery segment of at least 40 mm in length and without a reduction in lumen diameter >50%.
CAD, coronary artery disease; NIRS, Near-infrared spectroscopy; PCSK9, proprotein convertase subtilisin/kexin type 9.

in 203 patients with intracoronary NIRS imaging (NIRS cohort). For the long term follow-up, serum samples for PCSK9 measurements were available in 576 patients (full cohort). PCSK9 levels were measured in the stored serum samples using an enzyme linked immunosorbent assay (Human PCSK9 Quantikine ELISA, R&D systems Inc., Minneapolis, MN, USA). The minimum detectable level of this essay was 0.096 µg/L with a coefficient of variation of 4.1% at a mean value of 27.9 µg/L. The laboratory was blinded to clinical and intracoronary imaging data.

Near-infrared spectroscopy

Subsequent to the index CAG or PCI, invasive imaging with IVUS and NIRS was performed in one non-culprit coronary artery segment. The study protocol predefined the order of preference for the selection of the non-culprit study vessel: 1) left anterior descending artery; 2) right coronary artery; and 3) left circumflex artery. The non-culprit coronary artery segment had to be at least 40 mm in length and without a reduction in lumen diameter >50% by online angiographic visual assessment. The NIRS system, which was approved by the U.S. Food and Drug Administration, included a 3.2-F rapid exchange catheter, a pullback and rotation device, and a console (InfraReDx, Burlington, Massachusetts, USA). A motorized catheter pullback was performed at a speed of 0.5mm/s and 240rpm, starting distal to a side branch. Immediately after a pullback, the data in the scanned coronary artery segment were displayed in a chemogram. The probability of the presence of lipid rich core-containing plaques in the scanned coronary artery segment was calculated by means of a prediction algorithm and was displayed using colors, ranging from red (low probability of lipid content) to yellow-coded plaque (high probability of lipid content) (15)(Figure 2). LCBI was determined for the entire segment, as well as for the 10 mm and 4 mm long segments with the highest LCBI ($MaxLCBI_{4mm}$ and $MaxLCBI_{10mm}$). The NIRS images were analyzed offline by an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands) that was blinded to the clinical and PCSK9 data of the patients.

Follow-up and study endpoints

Clinical and vital status of patients were collected from medical charts, civil registries or by written or telephone contacts with the patients or relatives. Follow-up questionnaires as a screening tool for identifying probable adverse events were sent to all living patients participating in this study. For patients with any hospitalization

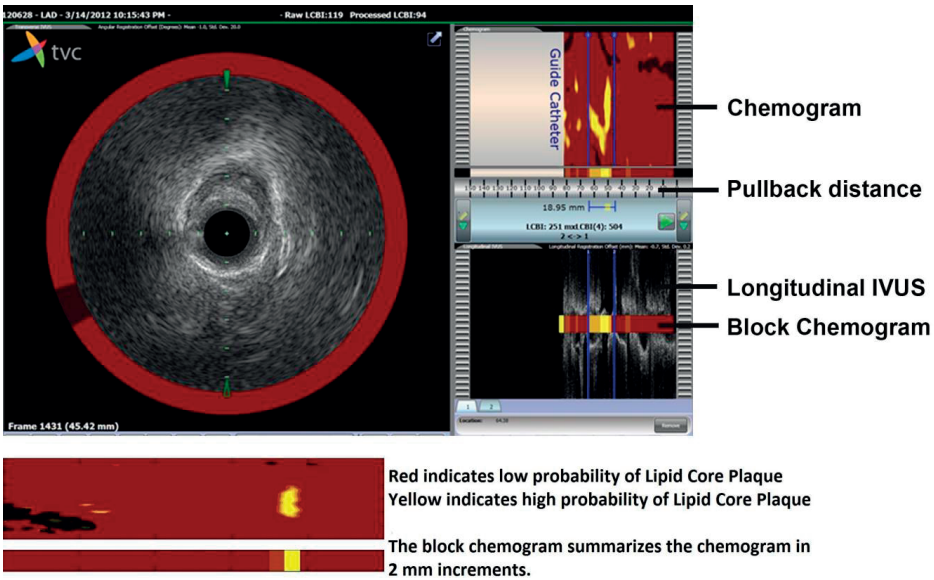


Figure 2. Intracoronary near-infrared spectroscopy
The figure displays an example of coronary wall imaging by NIRS. Spectral characteristics of lipid core plaques (LCP) are displayed on a chemogram along the length (x-axis, in mm) and circumference (y-axis, 0 to 360 degrees) of the scanned coronary artery. Yellow regions in the chemogram represent high probability of LCP while red regions represent those with low probability of LCP. The LCBI quantifies the amount of LCP in the entire scanned artery segment on the block chemogram, and is computed as the fraction of valid pixels that exceed an LCP probability of 0·6, multiplied with 1000. LCBI, Lipid Core Burden Index; NIRS, Near-infrared spectroscopy.

or a possible adverse event, additional information was obtained from hospital discharge letters.

The primary clinical endpoint was the occurrence of major adverse cardiac events (MACE), defined as the composite of all-cause mortality, nonfatal ACS or unplanned coronary revascularization. The secondary endpoint was the composite of all-cause mortality or nonfatal ACS. ACS was defined as the clinical diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris (UAP) in accordance with the guidelines of the European Society of Cardiology (16, 17). Unplanned coronary revascularization was defined as any PCI or coronary artery bypass grafting (CABG) that was not foreseen at the index procedure. Endpoints were adjudicated by a clinical events committee that was blinded to the serum PCSK9 levels and imaging data.

Statistical analysis

Categorical variables are presented as numbers and percentages. Normally-distributed continuous variables are presented as mean ± standard deviation (SD), while non-normally distributed continuous variables are presented as median with 25^{th}-75^{th} percentile. The distribution of continuous variables was examined for normality by visual inspection of the histogram. Serum PCSK9 levels (measured in µg/L) were not normally distributed and were therefore ln-transformed for further analyses (LnPCSK9). NIRS-derived LCBI, $MaxLCBI_{4mm}$, $MaxLCBI_{10mm}$ were first ln-transformed and then standardized as a z-score (Z_{lnLCBI}). Comparisons were done using the Chi-square test for categorical variables and Student's t-test and the Mann-Whitney U test for continuous variables.

Linear regression was used to examine the associations between PCSK9 levels (independent variable) and the NIRS findings (dependent variables) in the NIRS cohort (n= 203 patients). Results of linear regression are presented as the mean (95% confidence interval (CI)) change in Z_{lnLCBI} per unit change in lnPCSK9 level. We conducted multivariable analysis, including the following covariates: age, gender, hypertension, diabetes mellitus, LDL-C level, statin use at time of hospital admission and indication for index CAG.

Cumulative event rates were estimated according to the Kaplan-Meier method. Patients lost to follow-up were censored at the date of last contact. Cox proportional hazards models were used to evaluate the associations between PCSK9 levels and clinical study endpoints in the full cohort (n= 576 patients). PCSK9 level was analyzed as a categorical variable (serum PSCK9 levels above versus below the median) and as continuous variable. The final results are presented as unadjusted and multivariable adjusted hazard ratios (HRs) with 95% CIs.

To account for possible effect modification by baseline indication for CAG, all statistical analyses were performed in the overall study population with and without the interaction term on indication for CAG (i.e. SAP or ACS). Furthermore, stratified analysis by age, gender, diabetes, hypertension, hypercholesteremia, LDL-C level and statin use at hospital admission were also performed to assess effect modification. All statistical tests were two-tailed and p-values <0.05 were considered statistically significant. Data were analyzed using IBM SPSS software (SPSS 23.0 IBM corp., Armonk, NY, USA).

Table 1. Baseline clinical and procedural characteristics of the NIRS cohort

	Total (n=203)	ACS patients (n= 95)	SAP patients (n= 108)	P value
Clinical characteristics				
Age, years, mean ± SD	63.4 ± 10.9	62 ± 11.7	64.7 ± 10.2	0.082
Male, n (%)	148 (72.9)	63 (66.3)	85 (78.7)	0.048
Diabetes Mellitus, n (%)	41 (20.2)	17 (17.9)	24 (22.2)	0.443
Hypertension, n (%)	114 (56.2)	51 (53.7)	63 (58.3)	0.505
Hypercholesterolemia, n (%)	115 (56.7)	43 (45.3)	72 (66.7)	0.002
Smoking, n (%)	50 (24.6)	30 (31.6)	20(18.5)	0.067
Positive family history of CAD, n (%)	120 (59.1)	51 (54.3)	69 (63.9)	0.164
Previous MI, n (%)	79 (38.9)	34 (35.8)	45 (41.7)	0.391
Previous PCI, n (%)	78 (38.4)	27 (28.4)	51 (47.2)	0.006
Previous CABG, n (%)	6 (3.0)	2 (2.1)	4 (3.7)	0.502
Previous stroke, n (%)	6 (3.0)	4 (4.2)	2 (1.9)	0.322
Peripheral artery disease, n (%)	11 (5.4)	5 (5.3)	6 (5.6)	0.927
History of heart failure, n (%)	9 (5.9)	3 (3.2)	6 (5.6)	0.408
Serum PCSK 9 µg/L	278.3[217.5-343.9]	269.2[191.5-336.8]	280.4[222.4-358.7]	0.319
Statin use at baseline, n (%)	181 (89.2)	82 (86.3)	99 (91.7)	0.261
Serum TC mmol/L	4.20[3.60-4.90]	4.40[3.68-5.33]	4.00[3.40-4.80]	0.015
Serum LDL-C mmol/L	2.49[1.98-3.34]	2.82[2.08-3.60]	2.37[1.94-3.00]	0.007
Serum HDL-C mmol/L	1.06[0.87-1.33]	1.11[0.87-1.35]	1.06[0.87-1.31]	0.507
Serum TG mmol/L	1.30[0.93-1.87]	1.19[0.79-1.76]	1.42[1.08-2.10]	0.002
Procedural characteristics				
Indication for coronary angiography				
ACS, n (%)	95 (46.8)	95 (100)	0 (0)	
STEMI, n (%)	28 (13.8)	28 (29.5)	0 (0)	
Non ST-ACS/ UAP, n (%)	67 (33.0)	67 (70.5)	0 (0)	
Stable angina pectoris, n (%)	108 (53.2)	0 (0)	108 (100)	
PCI performed, n (%)	179 (88.2)	88 (92.6)	91 (84.3)	
Coronary artery disease [52]				
No significant stenosis, n (%)	16 (7.9)	8 (8.4)	8 (7.4)	
1-vessel disease, n (%)	106 (52.2)	49 (51.6)	57 (52.8)	
2-vessel disease, n (%)	58 (28.6)	26 (27.4)	32 (29.6)	
3-vessel disease, n (%)	23 (11.3)	12 (12.6)	11 (10.2)	
NIRS characteristics				
LCBI [25th-75th]	43.0[15.0-90.0]	47.0[16.0-90.0]	35.0[14.0-85.5]	0.441
LCBI $_{4mm}$ [25th-75th]	234[93-377]	267[100-387]	201[85-377]	0.431
LCBI $_{10mm}$[25th-75th]	131[60-247]	153[68-253]	239[47-121]	0.435
Imaged coronary artery				
Left anterior descending, n (%)	74 (36.5)	41 (43.2)	33 (30.6)	

Left circumflex, n (%)	70 (34.5)	30 (31.6)	40 (37.0)
Right coronary artery, n (%)	59 (29.1)	24 (25.3)	35 (32.4)

Continuous variables are presented as mean± standard deviation (SD) or as median with 25th-75th percentile. Categorical variables are presented in numbers and percentages n (%).
Continuous variables are presented as mean± standard deviation (SD) or as median with 25th-75th percentile. Categorical variables are presented in numbers and percentages n (%).
ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; HDL-C, high-density lipoprotein cholesterol; LCBI, Lipid Core Burden Index; LDL-C, low-density lipoprotein cholesterol; MI, myocardial infarction; NIRS, Near-infrared spectroscopy; Non ST-ACS, non ST segment elevation acute coronary syndrome ; PCI, percutaneous coronary intervention; PCSK9, proprotein convertase subtilisin/kexin type 9 SAP, stable angina pectoris; TG, triglycerides; UAP, unstable angina pectoris.

52 A significant stenosis was defined as a stenosis ≥ 50% of the vessel diameter by visual assessment of the coronary angiogram.

RESULTS

Baseline characteristics

A total of 203 patients were enrolled in the NIRS cohort (Figure 1). Mean age was 63.4 years and 72.9% were men (Table 1). The median PCSK9 level was 278 µg/L and ranged from 91 to 804 µg/L [25th-75th percentile; 218-344 µg/L] with no differences between patients admitted with ACS and patients with SAP. In contrast, the median serum LDL-C level was 2.49 [25th-75th percentile: 1.98-3.34] mmol/L and was higher in patients admitted with ACS when compared with patients with SAP (2.82 [2.08-3.60] versus 2.37 [1.94-3.00] mmol/l, p=0.007). At the time of hospital admission, statin use was 89.2%. A total of 46.8% of the patients were diagnosed with ACS. During the index CAG, PCI was performed in 88.2% of the patients. The median LCBI of the imaged coronary segment was 43 [25th-75th percentile: 15-90].

Association between PCSK9 level and NIRS-derived LCBI

Patients with higher serum PCSK9 levels also had higher NIRS-derived LCBI with a mean increase of 0.381 (95% CI [0.004-0.757]) in Z_{lnLCBI} per unit increase in lnPCSK9 level ('beta'; p=0.047; Table 2). This association remained statistically significant after multivariable adjustment for cardiac risk factors and statin use (Beta= 0.404; 95% CI [0.024-0.783], p=0.037), as well as after additional adjustment for baseline serum LDL-C level (Beta= 0.390 (95% CI [0.011-0.769] p =0.044)). Furthermore, in multivariable analysis, serum PCSK9 levels were also significantly associated with $LCBI_{10mm}$ (Beta= 0.406 (95% CI [0.014-0.797], p=0.042). Results were similar in patients admitted with ACS or SAP.

Table 2. Association between serum PCSK9 and LCBI

NIRS characteristics	Model1 Beta (95%CI)	P-value	Model 2 Beta (95%CI)	P-value	Model3 Beta (95%CI)	P-value
NIRS cohort (n=203)						
LCBI	0.381 (0.004-0.757)	0.047	0.404 (0.024-0.783)	0.037	0.390 (0.011-0.769)	0.044
LCBI $_{4\,mm}$	0.251 (-0.137-0.640)	0.203	0.292 (-0.099-0.684)	0.142	0.281 (-0.111-0.673)	0.159
LCBI $_{10\,mm}$	0.370 (-0.016-0.756)	0.060	0.411 (0.021-0.801)	0.039	0.406 (0.014-0.797)	0.042

The results are presented as beta coefficients (B) that indicate the mean (95% confidence interval (CI)) change in Z_{lnLCBI}, $Z_{lnLCBI4mm}$ or $Z_{lnLCBI10mm}$ per unit change in lnPCSK9.
Model 1 includes serum PCSK9 levels.
Model 2 includes serum PCSK9 levels, age, gender, diabetes mellitus, hypertension, indication for index CAG and statin use. [53]
Model 3 includes serum PCSK9 level, age, gender, diabetes mellitus, hypertension, indication for index CAG, statin use and serum LDL cholesterol.[54]
CAG, coronary angiography; LCBI, Lipid Core Burden Index; LDL, low-density lipoprotein; NIRS, Near-infrared spectroscopy; PCSK9, proprotein convertase subtilisin/kexin

Subgroup analyses are presented in Figure 3. A significant interaction for the association between serum PCSK9 levels and LCBI was found for statin use at hospital admission (p=< 0.001). In line with this, in patients without statin use at hospital admission, higher serum PCSK9 levels were significantly associated with a higher NIRS-derived LCBI. In patients that used statins at admission, no statistically significant association was found.

PCSK9 and cardiovascular outcome during long-term follow-up

During a median follow-up time of 4.7 [25th-75th percentile: 4.2-5.6] years, MACE occurred in 157 patients. In these patients, the median PCSK9 level was 283 µg/L [25th-75th percentile: 229-339 µg/L]. In patients without events, the median PCSK9 level was 263 µg/L [25th-75th percentile: 213-345 µg/L] p= 0.119.

The cumulative incidence of MACE, stratified according to PCSK9 levels, is depicted in Figure 4. Patients with serum PCSK9 levels above the median of 278 µg/L had significantly higher incidence of MACE (30% versus 23% at 4.7 years follow-up; HR [95%CI]: 1.42[1.03-1.94], p=0.031) as well as significantly higher incidence of death or ACS (21% versus 15 % at 4.7 years follow-up); HR [95%CI]: 1.50[1.01-2.21], p=0.044) than their counterparts with lower levels. After adjustment for cardiac risk factors, statin use and baseline serum LDL-C level, these associations persisted: patients with PCSK9 above the median had 53% higher incidence of MACE (HR

53 Statin use was registered at the time of hospital admission.
54 Serum LDL cholesterol concentration was measured prior to CAG.

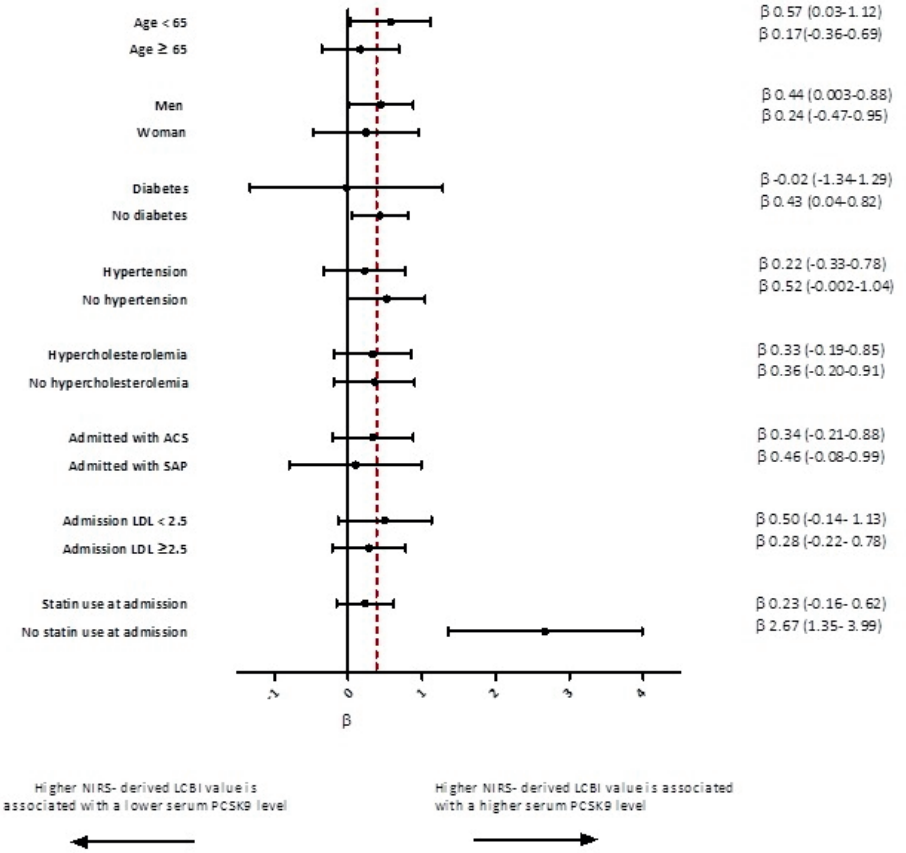


Figure 3. Association between serum PCSK9 concentration and NIRS-derived LCBI stratified by patient subgroups

Beta coefficients (B) indicate the mean (95% confidence interval (CI)) change in ZlnLCBI per unit change in lnPCSK9.

ACS, acute coronary syndrome; CAD, coronary artery disease; LCBI, Lipid Core Burden Index; LDL, low- density lipoprotein cholesterol level; NIRS, Near-infrared spectroscopy; PCSK9, proprotein convertase subtilisin/kexin type 9; SAP, stable angina pectoris.

[95%CI]: 1.53 [1.10-2.12], p=0.011), and 67% higher incidence of death or ACS (HR [95%CI]: 1.67 [1.12-2.50], p=0.013). Results were similar in SAP and ACS patients.

When serum PCSK9 level was analyzed as a continuous variable, and after multivariable adjustment, there was a tendency towards an association between serum PCSK9 levels and MACE (HR: 1.64; per unit increase in lnPCSK9 levels (95%CI [0.99-2.71], p=0.055)) and an association between PCSK9 levels and the composite

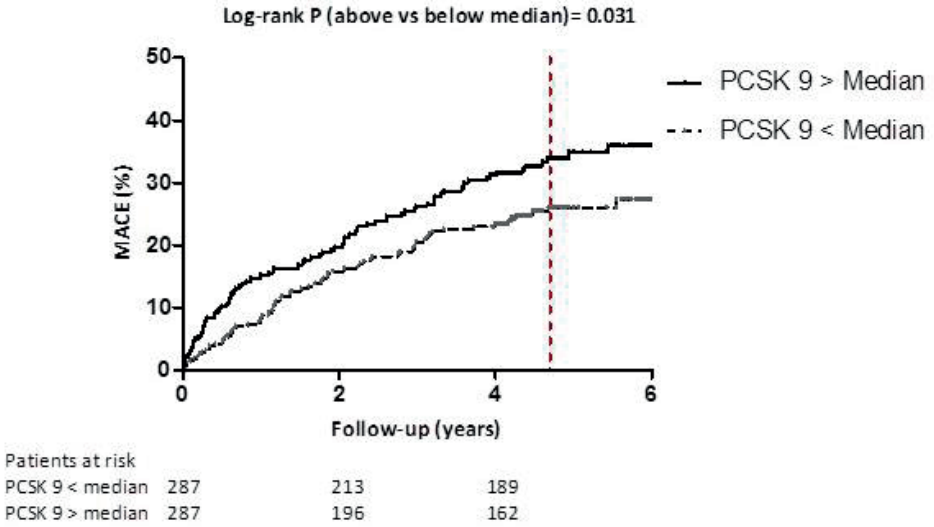


Figure 4. Association of PCSK9 level above vs below the median with clinical outcome in the full cohort (n=576).
MACE, major adverse cardiac events; PCSK9, proprotein convertase subtilisin/kexin type 9.

of death or ACS (HR: 1.88; per unit increase in lnPCSK9 levels 95%CI [1.01-3.51, p=0.047).

DISCUSSION

In patients undergoing CAG because of ACS or SAP, higher serum PCSK9 levels were associated with higher NIRS-derived LCBI. This association was independent of established cardiac risk factors, serum LDL-C level and statin use. Furthermore, serum PCSK9 levels were associated with the incidence of adverse cardiovascular outcomes as long as 4.7 years after the index procedure. Again, this association was independent of cardiac risk factors, serum LDL-C level and statin use.

The PCSK9 enzyme is a member of the proprotein convertase family of proteases, most closely related to proteinase-K. Previous genetic studies have shown that mutations in the PCSK9 gene are associated with either hypercholesterolemia with increased cardiovascular risk (gain-of-function mutations) or with hypocholesterolemia with decreased cardiovascular risk (loss-of-function mutations) (1-3). During the past decade, PCSK9 enzyme has been an intensively studied target for lipid lowering therapy in cardiovascular disease (4, 5, 7). It has been demonstrated that PCSK9 inhibitors suppress serum PCSK9 levels and consistently and substantially

reduce LDL-C levels (7). The FOURIER study showed that inhibition of PCSK9 on a background of statin therapy effectively decreased LDL-C levels and reduced the risk of cardiovascular events during long-term follow-up (8). Moreover, the GLAGOV study demonstrated that addition of PCSK9 inhibitors to stable statin therapy resulted in a greater decrease in plaque burden as assessed by IVUS (6). In our previous study in the current patient population, serum PCSK9 levels were associated with the fraction and amount of necrotic core tissue in coronary atherosclerotic plaques as assessed by IVUS (9). Our current finding that serum PCSK9 level is also associated with NIRS-derived LCBI extends and corroborates our previous findings. This association is independent of serum LDL-C level and statin use, which is important as statin treatment is known to increase PCSK9 levels by a negative feedback mechanism in reaction to lower cholesterol levels (24).

The PCSK9 enzyme induces the LDL-R degradation in the liver, resulting in an increase in circulating serum LDL-C levels that promote atherosclerosis (2). Therefore, it has been increasingly appreciated that PCSK9 plays a key role in the development of atherosclerosis through a lipid pathway. However, our current finding that serum PCSK9 levels were associated with NIRS-derived LCBI, independent of serum LDL-C levels, may also suggest a non-lipid pathway for serum PCSK9 levels in atherosclerosis. In fact, it is well known that inflammatory mechanisms play an important role in the pathophysiology of atherosclerosis and plaque vulnerability by mechanisms that are independent of LDL-C levels (18). In this respect, it is important to note that it has been shown that PCSK9 enzyme positively influences the expression of LOX-1 and mitochondrial reactive oxygen species (msROS), resulting in endothelial inflammation and damage (19, 20). In turn, msROS inhibition reduced the expression of both PCSK9 and LOX-1 (21). LOX-1 is a scavenger receptor in vascular cells and contributes to the development of atherosclerosis via increasing the uptake of oxidized-LDL (oxLDL), a major pro-inflammatory factor in atherosclerosis (20, 21). OxLDL and tumour necrosis factor-α (TNF-α) regulate PCSK9 expression that is mediated by the NF-κB signalling pathway (22). PCSK9 overexpression also upregulates TLR4 expression and promotes the activation of NF-kB (22). The TLR4/NF-kB signalling pathway is critical for atherogenesis since it regulates vascular inflammatory responses (22). PCSK9 may also increase the expression of VCAM-1 and ICAM-1 in endothelian cells and promote the adhesion of circulating inflammatory monocytes to the endothelium (19). Finally, previous studies have also demonstrated that serum PCSK9 levels were independently associated with higher levels of high-sensitivity CRP (hsCRP) and white blood cell count, both markers of

inflammation and mediators of atherosclerosis (23, 24). Our current finding, that serum PCSK9 levels are associated with NIRS-derived LCBI, independent of serum LDL-C levels, indirectly provides further support to these previous observations that serum PCSK9 levels contribute directly to the inflammation in the atherosclerotic plaque and may reflect the vulnerability of the entire coronary tree. Therefore, PCSK9 inhibition may exert its beneficial therapeutic effects not only by means of its LDL-C lowering, but also by its anti-inflammatory properties in CAD.

In prior studies, conflicting results have been observed on the relationship of serum PCSK9 levels with adverse cardiovascular outcome (23, 25-27). Although Gencer et al.(23) did not find a significant association between serum PCSK9 levels and 1-year follow-up, other studies found a significant association of serum PCSK9 levels with adverse cardiovascular outcome (25-27). Clinical studies in patients with CAD on the association of serum PCSK9 levels with cardiovascular outcome during long-term follow-up are scarce. Werner et al. demonstrated in a prospective observational study that serum PCSK9 levels predict cardiovascular outcome during 4-year follow-up in statin treated patients with stable CAD (27). The other studies have mostly used healthy populations during long-term follow-up (25, 26) or are with only short-term follow-up (23).

This study extends our previous 1-year follow-up data on the relationship between serum PCSK9 levels and adverse cardiac outcome in the ATHEROREMO study (9). The current study confirms these previous findings and extents these results to long-term follow-up. The adverse prognostic implications associated with higher PCSK9 levels may reflect the vulnerability of the entire coronary tree through a higher lipid content of coronary plaques as reflected by associated higher LCBI values, but also a more direct role in coronary plaque inflammation. The latter is supported by a recent analysis of the FOURIER study that showed that patients with higher hsCRP levels experienced a greater absolute risk reduction in cardiovascular events with the PCSK9 inhibitor evolocumab (28).

Some limitations of the present study need to be acknowledged. Firstly, by design of the study, repeated blood samples of serum PCSK9 levels and intracoronary NIRS imaging were not performed. Therefore, the effects of changes in serum PCSK9 levels and their effect on NIRS-derived LCBI over time could not be investigated. Secondly, NIRS imaging was limited to a pre-specified target segment of a non-culprit coronary artery. This method was chosen under the hypothesis that such a non-culprit coronary artery segment reflects the vulnerability of the entire coronary tree (14). In fact, we have indirectly supported this hypothesis in our previous studies by

showing that IVUS and NIRS imaging of the composition of coronary atherosclerosis in a non-culprit coronary artery segment predicts adverse outcome throughout the entire coronary tree (9, 12, 13). Finally, the NIRS chemogram only represents plaque information in a 2-dimensional manner and does not provide data on the depth of the cholesterol accumulation within the coronary artery wall. Nevertheless, it has previously been demonstrated that LCBI values obtained in a non-culprit coronary artery segment are strongly and independently associated with increased risk of cardiovascular outcome within the current study population (12, 13).

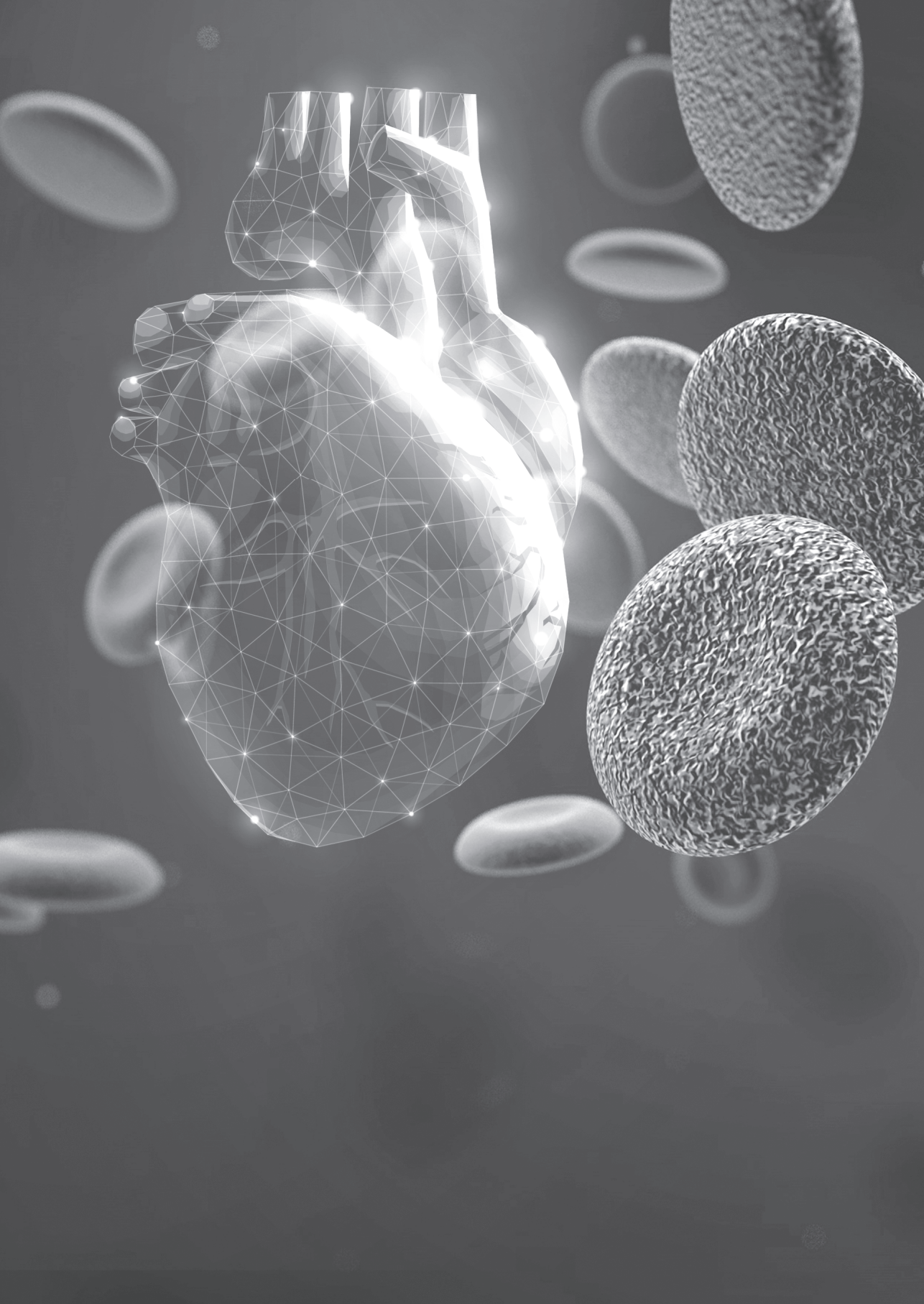
In conclusion, higher serum PCSK9 levels were associated with a higher NIRS-derived LCBI in a single non-culprit coronary artery segment in patients undergoing CAG because of ACS or SAP. This association was independent of established cardiac risk factors, as well as of serum LDL-C levels and statin use. Furthermore, serum PCSK9 levels were associated with the incidence of adverse cardiovascular outcomes during a median follow-up period of 4.7 years after the index procedure. Again this association was independent of cardiac risk factors, serum LDL-C levels and statin use.

REFERENCES

1. Urban D, Poss J, Bohm M, Laufs U. Targeting the proprotein convertase subtilisin/kexin type 9 for the treatment of dyslipidemia and atherosclerosis. J Am Coll Cardiol. 2013;62(16):1401-8.
2. Cohen JC, Boerwinkle E, Mosley TH, Jr., Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med. 2006;354(12):1264-72.
3. Kathiresan S, Myocardial Infarction Genetics C. A PCSK9 missense variant associated with a reduced risk of early-onset myocardial infarction. N Engl J Med. 2008;358(21):2299-300.
4. Robinson JG, Farnier M, Krempf M, Bergeron J, Luc G, Averna M, Stroes ES, Langslet G, Raal FJ, El Shahawy M, Koren MJ, Lepor NE, Lorenzato C, Pordy R, Chaudhari U, Kastelein JJ. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. N Engl J Med. 2015;372(16):1489-99.
5. Sabatine MS, Giugliano RP, Wiviott SD, Raal FJ, Blom DJ, Robinson J, Ballantyne CM, Somaratne R, Legg J, Wasserman SM, Scott R, Koren MJ, Stein EA. Efficacy and safety of evolocumab in reducing lipids and cardiovascular events. N Engl J Med. 2015;372(16):1500-9.
6. Nicholls SJ, Puri R, Anderson T, Ballantyne CM, Cho L, Kastelein JJ, Koenig W, Somaratne R, Kassahun H, Yang J, Wasserman SM, Scott R, Ungi I, Podolec J, Ophuis AO, Cornel JH, Borgman M, Brennan DM, Nissen SE. Effect of Evolocumab on Progression of Coronary Disease in Statin-Treated Patients: The GLAGOV Randomized Clinical Trial. Jama. 2016;316(22):2373-84.
7. Desai NR, Giugliano RP, Wasserman SM, Gibbs JP, Liu T, Scott R, Sabatine MS. Association Between Circulating Baseline Proprotein Convertase Subtilisin Kexin Type 9 Levels and Efficacy of Evolocumab. JAMA Cardiol. 2017;2(5):556-60.
8. Sabatine MS, Giugliano RP, Keech AC, Honarpour N, Wiviott SD, Murphy SA, Kuder JF, Wang H, Liu T, Wasserman SM, Sever PS, Pedersen TR. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. N Engl J Med. 2017;376(18):1713-22.
9. Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Boersma E, van Geuns RJ, Serruys PW, Kardys I, Akkerhuis KM. PCSK9 in relation to coronary plaque inflammation: Results of the ATHEROREMO-IVUS study. Atherosclerosis. 2016;248:117-22.
10. Waxman S, Dixon SR, L'Allier P, Moses JW, Petersen JL, Cutlip D, Tardif JC, Nesto RW, Muller JE, Hendricks MJ, Sum ST, Gardner CM, Goldstein JA, Stone GW, Krucoff MW. In vivo validation of a catheter-based near-infrared spectroscopy system for detection of lipid core coronary plaques: initial results of the SPECTACL study. JACC Cardiovasc Imaging. 2009;2(7):858-68.
11. Madder RD, Smith JL, Dixon SR, Goldstein JA. Composition of target lesions by near-infrared spectroscopy in patients with acute coronary syndrome versus stable angina. Circ Cardiovasc Interv. 2012;5(1):55-61.
12. Oemrawsingh RM, Cheng JM, Garcia-Garcia HM, van Geuns RJ, de Boer SP, Simsek C, Kardys I, Lenzen MJ, van Domburg RT, Regar E, Serruys PW, Akkerhuis KM, Boersma E. Near-infrared spectroscopy predicts cardiovascular outcome in patients with coronary artery disease. J Am Coll Cardiol. 2014;64(23):2510-8.

13. Schuurman AS, Vroegindewey M, Kardys I, Oemrawsingh RM, Cheng JM, de Boer S, Garcia-Garcia HM, van Geuns RJ, Regar ES, Daemen J, van Mieghem NM, Serruys PW, Boersma E, Akkerhuis KM. Near-infrared spectroscopy-derived lipid core burden index predicts adverse cardiovascular outcome in patients with coronary artery disease during long-term follow-up. Eur Heart J. 2018;39(4):295-302.
14. de Boer SP, Cheng JM, Garcia-Garcia HM, Oemrawsingh RM, van Geuns RJ, Regar E, Zijlstra F, Laaksonen R, Halperin E, Kleber ME, Koenig W, Boersma E, Serruys PW. 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(8):953-60.
15. Brugaletta S, Garcia-Garcia HM, Serruys PW, de Boer S, Ligthart J, Gomez-Lara J, Witberg K, Diletti R, Wykrzykowska J, van Geuns RJ, Schultz C, Regar E, Duckers HJ, van Mieghem N, de Jaegere P, Madden SP, Muller JE, van der Steen AF, van der Giessen WJ, Boersma E. NIRS and IVUS for characterization of atherosclerosis in patients undergoing coronary angiography. JACC Cardiovasc Imaging. 2011;4(6):647-55.
16. Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). Eur Heart J. 2016;37(3):267-315.
17. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J. 2017.
18. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002;105(9):1135-43.
19. Tang ZH, Li TH, Peng J, Zheng J, Li TT, Liu LS, Jiang ZS, Zheng XL. PCSK9: A novel inflammation modulator in atherosclerosis? J Cell Physiol. 2019;234(3):2345-55.
20. Ding Z, Liu S, Wang X, Deng X, Fan Y, Shahanawaz J, Shmookler Reis RJ, Varughese KI, Sawamura T, Mehta JL. Cross-talk between LOX-1 and PCSK9 in vascular tissues. Cardiovasc Res. 2015;107(4):556-67.
21. Tang Z, Jiang L, Peng J, Ren Z, Wei D, Wu C, Pan L, Jiang Z, Liu L. PCSK9 siRNA suppresses the inflammatory response induced by oxLDL through inhibition of NF-kappaB activation in THP-1-derived macrophages. Int J Mol Med. 2012;30(4):931-8.
22. Tang ZH, Peng J, Ren Z, Yang J, Li TT, Li TH, Wang Z, Wei DH, Liu LS, Zheng XL, Jiang ZS. New role of PCSK9 in atherosclerotic inflammation promotion involving the TLR4/NF-kappaB pathway. Atherosclerosis. 2017;262:113-22.
23. Gencer B, Montecucco F, Nanchen D, Carbone F, Klingenberg R, Vuilleumier N, Aghlmandi S, Heg D, Räber L, Auer R, Jüni P, Windecker S, Lüscher TF, Matter CM, Rodondi N, Mach F. Prognostic value of PCSK9 levels in patients with acute coronary syndromes. Eur Heart J. 2016;37(6):546-53.
24. Li S, Guo YL, Xu RX, Zhang Y, Zhu CG, Sun J, Qing P, Wu NQ, Jiang LX, Li JJ. Association of plasma PCSK9 levels with white blood cell count and its subsets in patients with stable coronary artery disease. Atherosclerosis. 2014;234(2):441-5.

25. Leander K, Malarstig A, Van't Hooft FM, Hyde C, Hellenius ML, Troutt JS, Konrad RJ, Öhrvik J, Hamsten A, de Faire U. Circulating Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Predicts Future Risk of Cardiovascular Events Independently of Established Risk Factors. Circulation. 2016;133(13):1230-9.
26. Vlachopoulos C, Terentes-Printzios D, Georgiopoulos G, Skoumas I, Koutagiar I, Ioakeimidis N, Stefanadis C, Tousoulis D. Prediction of cardiovascular events with levels of proprotein convertase subtilisin/kexin type 9: A systematic review and meta-analysis. Atherosclerosis. 2016;252:50-60.
27. Werner C, Hoffmann MM, Winkler K, Bohm M, Laufs U. Risk prediction with proprotein convertase subtilisin/kexin type 9 (PCSK9) in patients with stable coronary disease on statin treatment. Vascul Pharmacol. 2014;62(2):94-102.
28. Bohula EA, Giugliano RP, Leiter LA, Verma S, Park JG, Sever PS, Lira Pineda A, Honarpour N, Wang H, Murphy SA, Keech A, Pedersen TR, Sabatine MS. Inflammatory and Cholesterol Risk in the FOURIER Trial. Circulation. 2018;138(2):131-40.



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Changes of C-reactive protein levels and coronary plaque composition after intensive statin therapy in patients with coronary artery disease (IBIS-3 study)

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Changes of C-reactive protein levels and coronary plaque composition after intensive statin therapy in patients with coronary artery disease (IBIS-3 study)

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Submitted

ABSTRACT

***Objective**:* To study the association between changes in serum C-reactive protein (CRP), (low density lipoprotein [LDL]) cholesterol and coronary plaque characteristics (based on invasive imaging) after 12 months of high dose rosuvastatin treatment in patients with established coronary artery disease (CAD).

***Methods**:* The IBIS-3 study is an observational study. A total of 164 patients undergoing coronary angiography (CAG) or percutaneous coronary intervention (PCI) for stable angina pectoris (SAP, N=96) or acute coronary syndrome (ACS, N=68) were treated with a high dose of rosuvastatin (40 mg daily) for 12 months. During the index procedure, intravascular ultrasound (IVUS) imaging of a non-culprit coronary segment was performed, whereas near-infrared spectroscopy (NIRS) of the same segment was performed in a subset of 118 patients. Blood samples for biomarker analyses were obtained immediately prior to the index procedure. At the end of the scheduled rosuvastatin treatment period, intracoronary imaging of the same segment and blood sampling were repeated.

***Results**:* Median (interquartile range) baseline and follow-up CRP-levels were 2.10 (0.85, 5.35) mg/L and 1.00 (0.50, 2.20) mg/L, respectively. The median change (Δ, follow-up minus baseline) in CRP was -0.45 (-2.55, 0.00) mg/L (p-value <0.001). We found no relevant differences in baseline clinical and imaging characteristics in relation to ΔCRP, except for the indication of the index procedure. Changes in CRP levels appeared considerably smaller in SAP (-0.20 [-1.10, 0.05] mg/L) than in ACS patients (-1.60 [-6.35, 0.00] mg/L). LDL-C was significantly decreased during 1-year rosuvastatin treatment, but ΔCRP was uncorrelated with ΔLCL-C (Spearman correlaton coefficient [$r_{spearman}$] -0.053 and

-0.060 in SAP and ACS, respectively). In ACS patients, but not SAP, ΔCRP was associated with IVUS-derived Δtotal plaque volume ($r_{spearman}$ 0.427), Δplaque burden ($r_{spearman}$ 0.336) and Δnecrotic core volume ($r_{spearman}$ 0.375). ΔCRP and NIRS-derived Δlipid core burden index were uncorrelated.

Conclusion: After 1 year intensive rosuvastatin therapy clinically relevant reductions in CRP levels were observed in a series of patients with established CAD. The observed CRP changes were correlated with changes in IVUS-derived plaque characteristics in ACS patients, but not in SAP. CRP changes were uncorrelated with changes in LDL-C levels. Hence, our study supports the role of in inflammation in CAD progression, but also emphasizes that the relation between blood cholesterol (LDL-C), inflammation (CRP), and extent and composition of coronary plaques is not straightforward.

INTRODUCTION

Inflammation is known to play a major role in the initiation, progression, and (in)stability of atherosclerotic plaques (1, 2). Against this background, C-reactive protein (CRP), a widely accessible, non-specific inflammatory biomarker, has been studied and proven as a risk factor for and prognostic factor in coronary artery disease (CAD) (3-7). Moreover, the recent CANTOS trial underscored the role of inflammation in the causal pathway of CAD development, as a reduction of CRP levels by canakinumab was directly accompanied by a reduction in the incidence of cardiovascular events, whereas low-density lipoprotein cholesterol (LDL-C) was not affected (4,5). The JUPITER study showed that a significant reduction of serum CRP levels can be realised by (rosuva)statin treatment in subjects with increased CAD risk (3). In JUPITER, the observed reductions in CRP (from a median of 4.2 mg/L at baseline to 1.8 mg/L at 4 year follow-up) and LDL-C levels (from 2.80 to 1.4 mmol/L), were associated with an important reduction in major adverse cardiovascular events (MACE) from 1.36 to 0.77 per 100 person-years of follow-up. On-treatment CRP levels were also associated with MACE in the SATURN study of intensive rosuvastatin or atorvastatin treatment in patients with established CAD (8). In parallel, the SATURN trialists demonstrated that the studied statin regimens resulted in a significant regression of coronary atherosclerosis, as assessed by intravascular ultrasound (IVUS) imaging at 2 year follow-up (9).

We designed the third Integrated Biomarker and Imaging Study (IBIS-3) to evaluate the effect of high-intensity (intended dose: 40 mg/day) rosuvastatin treatment for 1 year on coronary plaque characteristics in CAD patients, as assessed by multiple intravascular imaging modalities (10). Serial (radiofrequency [RF]) IVUS measurements were performed to study changes in total plaque volume and necrotic core (NC) volume, whereas near-infrared spectroscopy (NIRS) was applied to study changes in the lipid core burden index (LCBI). In IBIS-3, we observed a significant 30% reduction in LDL-C level (from a median of 2.36 mmol/L at baseline to 1.60 mmol/L at 1 year), but no reduction in NC volume (from 17.8 to 19.2 mm^3) or LCBI (from 183 to 192 in the 4 mm section with the highest values at baseline) of the study segment (11). We now studied changes in CRP levels, which we correlated with lipid levels and intracoronary imaging findings.

METHODS

Design

The IBIS-3 design details have been described elsewhere (9). Briefly, a total of 164 patients undergoing coronary angiography (CAG) or percutaneous coronary intervention (PCI) for stable angina pectoris (SAP, N=96) or acute coronary syndrome (ACS, N=68) were treated with a high dose of rosuvastatin (40 mg daily) for 12 months. During the index procedure, RF-IVUS of a non-culprit coronary segment was performed, whereas near-infrarad spectroscopy (NIRS) of the same segment was performed in a subset of 118 patients. Blood samples for biomarker analyses were obtained immediately prior to the index procedure. At the end of the scheduled rosuvastatin treatment period, intracoronary imaging of the same segment and blood sampling were repeated.

The IBIS-3 protocol was approved by the medical ethics committee of the Erasmus MC. Written informed consent was obtained from all included patients.

Intravascular imaging

Subsequent to the index procedure, invasive imaging was performed in a non-culprit coronary artery segment. This segment had to be at least 40 mm in length and without a reduction in lumen diameter >50% by online angiographic visual assessment. RF-IVUS imaging was performed with the Eagle-Eye catheter (Volcano Corporation, San Diego, CA, USA). NIRS imaging was performed with the infraredx system (InfraReDx, Burlington, Massachusetts, USA). Similar procedures were performed to

image the study segment during the follow-up visit. The RF-IVUS and NIRS images were analysed offline by an independent core research laboratory (Cardialysis BV, Rotterdam, the Netherlands) that was blinded for the timing of the measurements (baseline or follow-up), as well as for clinical and biomarker data.

Blood sampling and CRP measurements

Immediately prior to the index procedure and immediately prior to the follow-up CAG, blood samples were collected from the arterial sheath, which were transported to the clinical laboratory of the Erasmus MC within 2h for storage at -80°C. After completion of the study, high sensitivity CRP levels were batch-wise determined by using an immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Cobas 8000 modular analyser platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland).

Study endpoints

We determined changes in CRP, cholesterol (LDL-C, HDL-C and total cholesterol [Total-C]), IVUS-derived NC volume, and NIRS-derived LCBI for the entire region of interest (ROI) ($LCBI_{ROI}$), and the 10 mm ($LCBI_{10mm}$) and 4 mm ($LCBI_{4mm}$) sections with the highest LCBI. Changes in study endpoints are reported as measurements at follow-up minus baseline values. Hence, negative values indicate a decrease over time.

Data analyses

Categorical variables are reported as numbers and percentages. Continuous variables with a normal distribution are reported as mean ± standard deviation (SD), and otherwise as median with interquartile range (IQR). Patients were stratified in tertiles according to the observed change in CRP levels. Differences in baseline clinical characteristics between these strata were evaluated by chi-square or Fisher's exact tests for categorical variables and one-way analysis of variance (ANOVA) or Kruskal-Wallis tests for continuous variables.

Correlation analyses were performed to examine the associations between changes in CRP levels and changes in cholesterol levels and intracoronary imaging characteristics. Results are presented as Spearman correlation coefficients ($r_{spearman}$). We applied (multiple) linear regression analyses to relate (changes in) CRP level with (changes in) imaging characteristics. We considered age, sex, CVD risk factors, CVD history, and the extend of CAD as potential confounders. We ran separate

analyses for SAP and ACS, since the changes in CRP levels were considerably different between these patients (see the Results section).

All statistical tests were two-tailed and p-values <0.05 were considered significant. Data were analysed with SPSS software (SPSS 23.0 IBM corp., Armonk, NY, USA).

RESULTS

Baseline and follow-up CRP levels

Median (IQR) baseline and follow-up CRP-levels were 2.10 (0.85, 5.35) mg/L and 1.00 (0.50, 2.20) mg/L, respectively. The median change in CRP was -0.45 (-2.55, 0.00) mg/L, which was statistically significant (sign test p-value <0.001). We found no relevant differences in baseline clinical (Table 1) and imaging (Table 2) characteristics in relation to CRP change, except for the indication of the index procedure. Changes in CRP levels appeared considerably smaller in SAP than in ACS patients. SAP patients had median baseline and follow-up CRP of 1.35 (0.70 to 4.25) mg/L and 0.90 (0.50, 2.55) mg/L, respectively, with a median change of -0.20 (-1.10, 0.05) mg/L (Table 3; Figure 1). ACS patients had median baseline and follow-up CRP of 2.80 (1.25 to 7.85) mg/L and 1.00 (0.40, 2.20) mg/L, respectively, with a median change of -1.60 (-6.35, 0.00) mg/L.

CRP and cholesterol

Baseline CRP and LDL-C were uncorrelated in SAP as well as ACS patients ($r_{spearman}$ -0.030 and -0.141, respectively), and so was Total-C (Figure 2). In SAP patients, high CRP levels were associated with low HDL-C ($r_{spearman}$ -0.325, p-value 0.001). Significant changes were observed during 1-year rosuvastatin treatment for LDL-C (decrease), HDL-C (increase) and Total-C values (decrease) in SAP and ACS patients, which, however, were not correlated with changes in CRP level (Figure 3).

CRP and IVUS characteristics

Baseline CRP and IVUS plaque characteristics were uncorrelated (Figure 4). No systematic changes were observed in total plaque volume and plaque burden during the 1-year treatment with rosuvastatin in SAP patients. In contrast, in ACS patients, total plaque volume (+6.8 mm^3; p-value 0.068) and plaque burden (+1.35%; p-value 0.005) tended to increase. NC volume decreased in SAP and ACS patients (-0.55 mm^3 and -0.31 mm^3, respectively). In ACS patients, not SAP, changes in CRP and changes in IVUS plaque characteristics were positively correlated (Figure 5). In

Table 1. Clinical baseline characteristics of the study patients, stratified by tertile of change in CRP level

	ΔCRP ≤ -1.6 *	-1.6 < ΔCRP < 0	ΔCRP ≥ 0	P-value †
Number of patients	54	50	60	
Age, years	57.4 (9.4)	60.4 (9.2)	61.2 (8.1)	0.108
Male	47 (87.0)	40 (80.0)	51 (85.0)	0.617
History of diabetes mellitus	12 (22.2)	12 (24.0)	11 (18.3)	0.744
History of hypertension	35 (64.8)	29 (58.0)	40 (69.0)	0.483
History of hypercholesterolemia	31 (57.4)	32 (64.0)	41 (70.7)	0.355
Baseline cholesterol measurements				
LDL-C, mmol/L	2.42 [1.82-3.02]	2.25 [1.88-2.86]	2.39 [1.93-2.87]	0.789
HDL-C, mmol/L	1.02 [0.86-1.17]	1.07 [0.89-1.28]	1.22 [0.93-1.39]	0.013
Total cholesterol, mmol/L	4.00 [3.30-5.00]	3.90 [3.20-4.60]	3.95 [3.50-4.60]	0.690
Current smoker	18 (33.3)	15 (30.0)	13 (21.7)	0.370
Positive family history of CAD	30 (56.6)	28 (56.0)	31 (51.7)	0.867
Previous MI	18 (33.3)	13 (26.0)	19 (31.7)	0.736
Previous PCI	23 (42.6)	14 (28.0)	23 (38.3)	0.291
Previous CABG	0	0	1 (1.7)	--
Previous stroke	3 (5.6)	4 (8.0)	8 (13.3)	0.386
Peripheral artery disease	1 (1.9)	4 (8.0)	2 (3.3)	0.299
History of heart failure	0	1 (2.0)	1 (1.7)	0.758
Statin use at baseline	51 (94.4)	48 (96.0)	57 (95.0)	1.000
PCI performed after index CAG	49 (90.7)	45 (90.0)	52 (86.7)	0.817
Indication for coronary angiography				<0.001
Stable angina pectoris	20 (37.0)	35 (70.0)	41 (68.3)	
ACS	34 (63.0)	15 (30.0)	19 (31.7)	

Categorical variables are presented as numbers and percentages n (%). Continuous variables are presented as mean (standard deviation) or median (interquartile range).

ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CRP, c-reactive protein; HDL, high-density lipoprotein; IQR, interquartile range; LCBI, Lipid Core Burden Index; LDL, low-density lipoprotein; MI, myocardial infarction; NC, necrotic core volume; NIRS, Near-infrared spectroscopy; PCI, percutaneous coronary intervention; SAP, stable angina pectoris

* ΔCRP, follow-up minus baseline CRP level

† Based on the Fisher's exact test (categorical data) or the Kruskal-Wallis test (continuous data)

particular, 13 (68%) of the 19 ACS patients with a delta CRP ≥0 mg/L had a delta NC volume ≥0 mm^3, as compared with 20 (41%) of the 49 patients with delta CRP <0 mg/L (p-value 0.059). In a linear regression model, each 1 mg/L difference in delta CRP was associated with 0.22 mm^3 difference in delta NC volume (p-value 0.010), after adjustment for baseline NC volume, sex, previous myocardial infarction, delta LDL-C (which appeared significant predictors in multivariable analysis) and timing of the reCAG. Also, 79% of the ACS patients with delta CRP ≥0 mg/L had

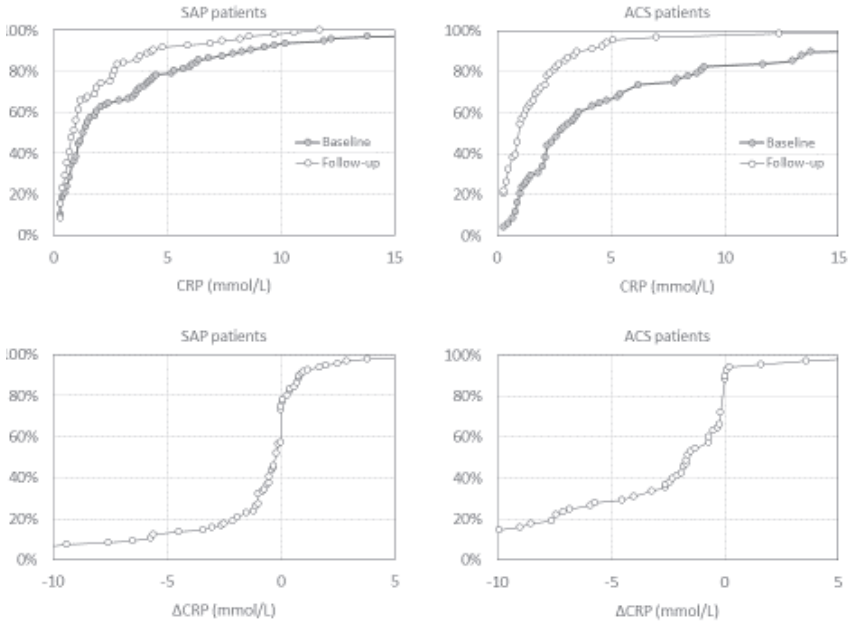
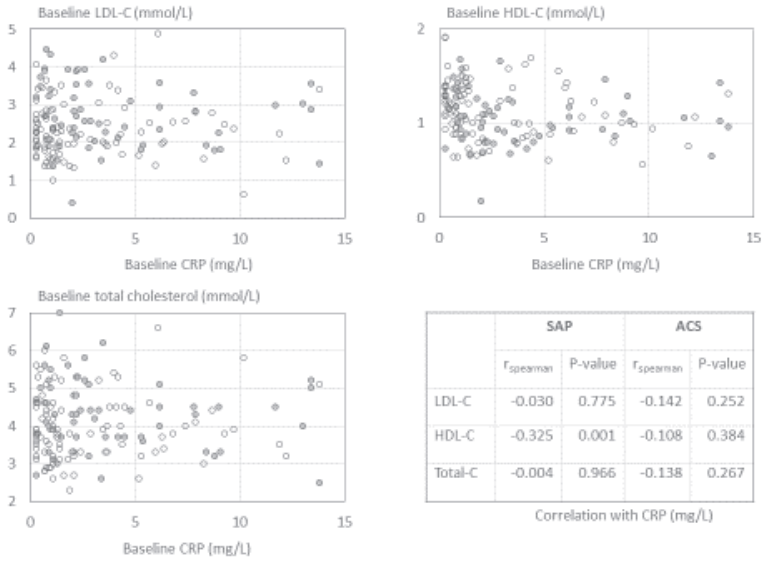


Figure 1 Cumulative distribution of baseline and follow CRP levels, as well as delta (Δ, follow-up minus baseline) CRP, in patients presenting with stable angina pectoris and acute coronary syndrome



	SAP		ACS	
	$r_{spearman}$	P-value	$r_{spearman}$	P-value
LDL-C	-0.030	0.775	-0.142	0.252
HDL-C	-0.325	0.001	-0.108	0.384
Total-C	-0.004	0.966	-0.138	0.267

Correlation with CRP (mg/L)

Figure 2 Relation between baseline CRP and baseline LDL-C, HDL-C and total cholesterol levels in patients presenting with stable angina pectoris (SAP, open bullet points) and acute coronary syndrome (ACS, closed bullet points)

Table 2. Imaging characteristics of the study patients, stratified by tertile of change in CRP level

	ΔCRP ≤ -1.6 *	-1.6 < ΔCRP < 0	ΔCRP ≥ 0	P-value †
Coronary artery disease ‡				0.645
Number of patients	54	50	60	
No significant stenosis	0	3 (6.0)	3 (5.0)	
1-vessel disease	30 (55.6)	24 (48.0)	31 (51.7)	
2-vessel disease	20 (37.0)	21 (42.0)	22 (36.7)	
3-vessel disease	4 (7.4)	2 (4.0)	4 (6.7)	
NIRS measurements				
Number of patients	35	31	37	
$LCBI_{ROI}$	29 [5-84]	36 [5-61]	40 [10-71]	0.701
$LCBI_{10mm}$	118 [9-223]	90 [10-181]	112 [28-207]	0.680
$LCBI_{4mm}$	189 [11-339]	178 [26-319]	182 [66-301]	0.921
IVUS measurements				
Number of patients	54	50	60	
Plaque volume, mm^3	201 [135-299]	190 [142-327]	217 [137-298]	0.866
Plaque burden (%)	42 [32-51]	39 [33-47]	41 [34-49]	0.779
NC volume, mm^3	17 [7-45]	13 [7-37]	20 [7-34]	0.778

Categorical variables are presented as numbers and percentages n (%). Continuous variables are presented as median (interquartile range).

ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CRP, c-reactive protein; HDL, high-density lipoprotein; IQR, interquartile range; LCBI, Lipid Core Burden Index; LDL, low-density lipoprotein; MI, myocardial infarction; NC, necrotic core volume; NIRS, Near-infrared spectroscopy; PCI, percutaneous coronary intervention; SAP, stable angina pectoris

* ΔCRP, follow-up minus baseline CRP level (mmol/L)

† Based on the Fisher's exact test (categorical data) or the Kruskal-Wallis test (continuous data)

‡ A significant stenosis was defined as a stenosis ≥ 50% of the vessel diameter by visual assessment of the coronary angiogram.

a delta total plaque volume ≥0 mm^3, compared with 55% of those with delta CRP <0 mg/L (p-value 0.097). Each 1 mg/L difference in delta CRP was associated with 0.81 mm^3 difference in delta total plaque volume (p-value 0.002), after adjustment for baseline total plaque volume (significant predictor) and timing of the reCAG. Finally, a 1mg/L difference in delta CRP was related with a 0.13 percent point difference in plaque burden, after adjustment for baseline plaque burden, age, previous PCI (significant predictors), and timing of the reCAG.

CRP and NIRS characteristics

Baseline CRP and $LCBI_{ROI}$, $LCBI_{10mm}$, $LCBI_{4mm}$ were also uncorrelated (Figure 6). LCBI values did not systematically change during the 1-year treatment period. For ex-

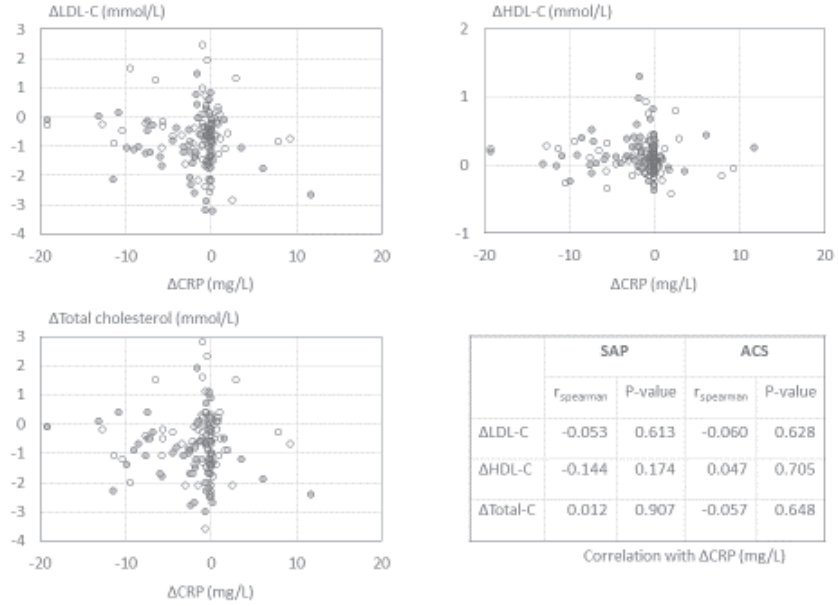
Table 3. Baseline and follow-up cholesterol and intracoronary imaging endpoints

	SAP				ACS			
	Baseline	Follow-up	Delta	P-value *	Baseline	Follow-up	Delta	P-value *
CRP, mg/L	1.35 (0.70, 4.25)	0.90 (0.50, 2.55)	-0.20 (-1.10, 0.05)	0.002	2.80 (1.25, 7.85)	1.00 (0.40, 2.20)	-1.60 (-6.35, 0.00)	<0.001
LDL-C, mmol/L	2.27 (1.88, 2.78)	1.62 (1.23, 2.11)	-0.62 (-1.08, -0.23)	<0.001	2.56 (2.03, 3.31)	1.59 (1.27, 1.92)	-1.03 (-1.67, -0.38)	<0.001
HDL-C, mmol/L	1.10 (0.91, 1.37)	1.14 (0.95, 1.44)	0.07 (-0.06, 0.20)	0.035	1.06 (0.86, 1.26)	1.21 (0.99, 1.46)	0.12 (0.02, 0.34)	<0.001
Total cholesterol, mmol/L	3.80 (3.30, 4.50)	3.30 (2.80, 3.80)	-0.60 (-1.30, -0.10)	<0.001	4.30 (3.70, 5.00)	3.20 (2.70, 3.50)	-0.95 (-1.70, -0.50)	<0.001
Plaque volume, mm^3	225 (137, 341)	231 (140, 343)	1.9 (-8.6, 16.2)	0.358	181 (144, 244)	197 (147, 252)	6.8 (-9.0, 18.2)	0.068
Plaque burden (%)	41.8 (32.8, 49.0)	41.2 (33.6, 50.3)	0.50 (-1.52, 2.80)	0.262	39 (33, 47)	41.4 (33.8, 48.0)	1.35 (-0.39, 3.40)	0.005
NC volume, mm^3	20.6 (7.4, 38.9)	19.8 (6.0, 37.4)	-0.55 (-4.27, 1.85)	0.064	16 (7, 35)	17.1 (6.0, 30.6)	-0.31 (-4.63, 1.65)	0.807
$LCBI_{ROI}$	29 (7, 64)	33 (9, 68)	0 (-16, 22)	0.798	34 (4, 73)	42 (7, 79)	5 (-22, 31)	0.133
$LCBI_{10mm}$	107 (27, 183)	106 (37, 199)	0 (-52, 69)	1.000	109 (22, 223)	119 (16, 181)	0 (-57, 50)	1.000
$LCBI_{4mm}$	183 (63, 315)	196 (89, 335)	10 (-68, 103)	0.162	184 (54, 332)	190 (39, 321)	8 (-32, 56)	0.405

Data are presented as median (interquartile range).

ACS, acute coronary syndrome; CRP, c-reactive protein; HDL, high-density lipoprotein; LCBI, Lipid Core Burden Index; LDL, low-density lipoprotein; NC, necrotic core; SAP, stable angina pectoris

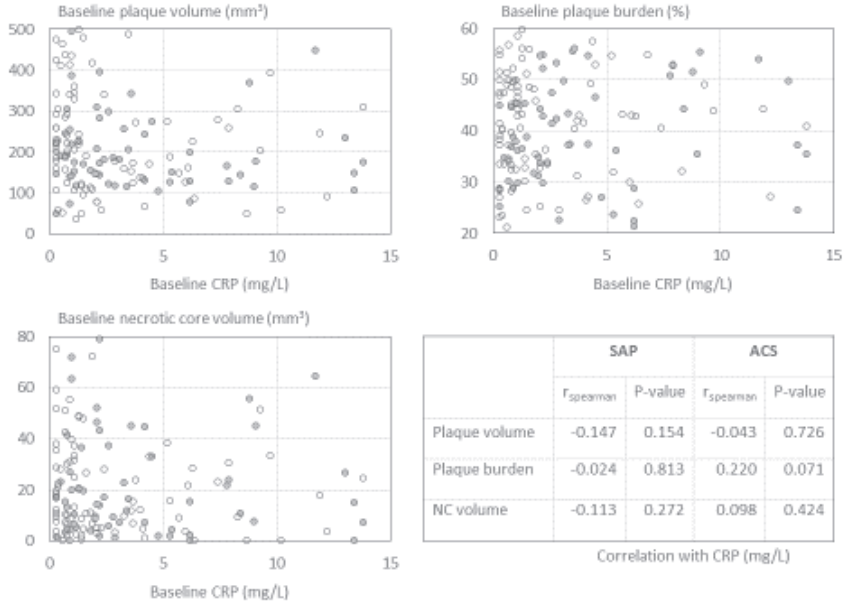
* P-value for change, based on the sign test



	SAP		ACS	
	$r_{spearman}$	P-value	$r_{spearman}$	P-value
ΔLDL-C	-0.053	0.613	-0.060	0.628
ΔHDL-C	-0.144	0.174	0.047	0.705
ΔTotal-C	0.012	0.907	-0.057	0.648

Correlation with ΔCRP (mg/L)

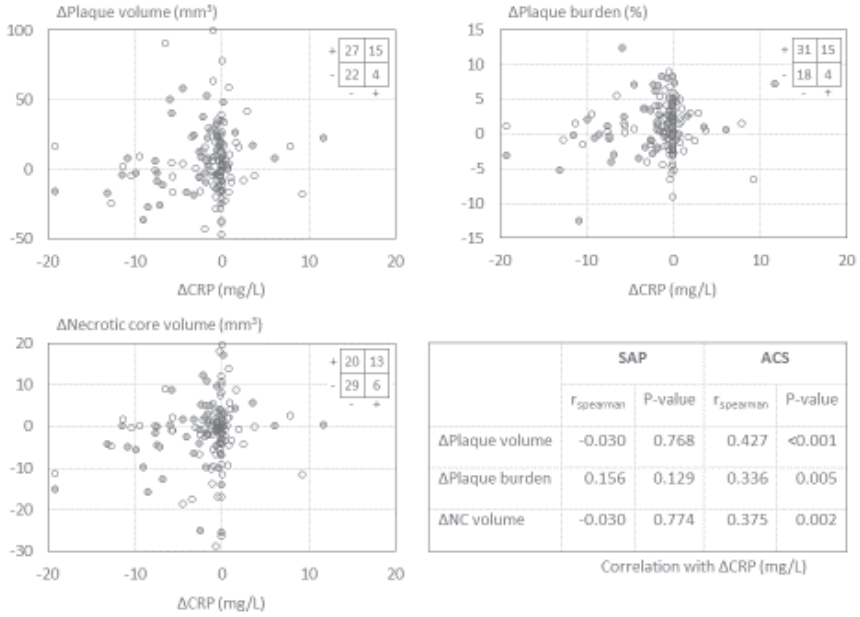
Figure 3 Relation between changes (Δ, follow-up minus baseline) in CRP and changes in LDL-C, HDL-C and total cholesterol levels in patients presenting with stable angina pectoris (SAP, open bullet points) and acute coronary syndrome (ACS, closed bullet points)



	SAP		ACS	
	$r_{spearman}$	P-value	$r_{spearman}$	P-value
Plaque volume	-0.147	0.154	-0.043	0.726
Plaque burden	-0.024	0.813	0.220	0.071
NC volume	-0.113	0.272	0.098	0.424

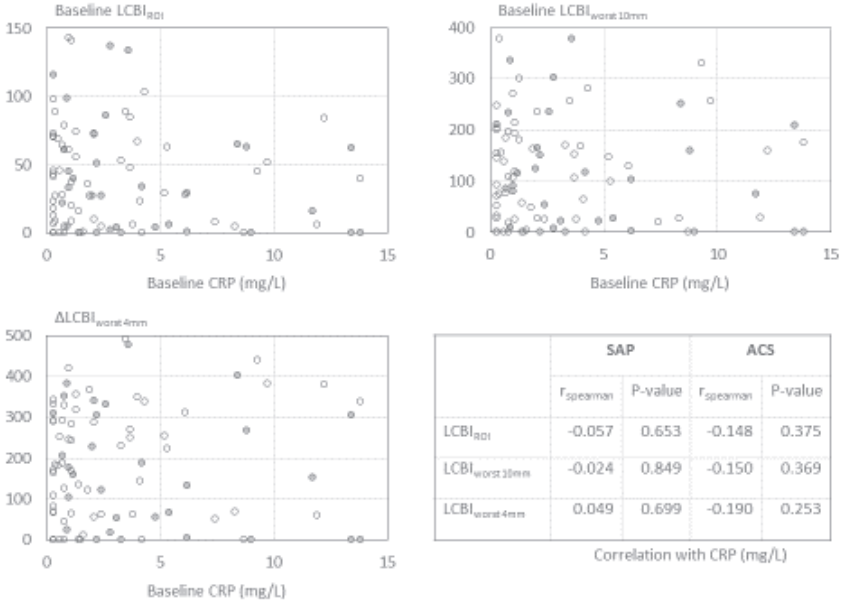
Correlation with CRP (mg/L)

Figure 4 Relation between baseline CRP and baseline total plaque volume, total plaque burden and necrotic core (NC) volume in patients presenting with stable angina pectoris (SAP, open bullet points) and acute coronary syndrome (ACS, closed bullet points)



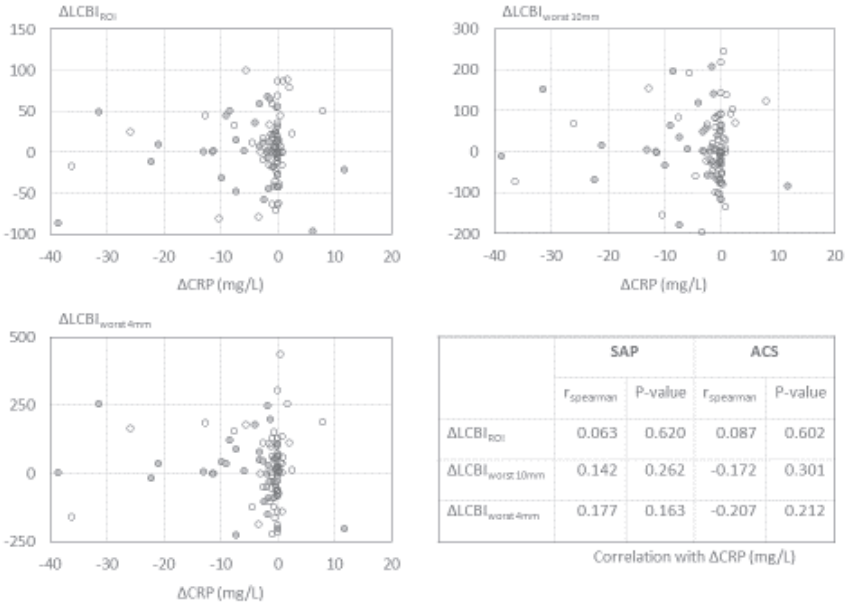
	SAP		ACS	
	$r_{spearman}$	P-value	$r_{spearman}$	P-value
ΔPlaque volume	-0.030	0.768	0.427	<0.001
ΔPlaque burden	0.156	0.129	0.336	0.005
ΔNC volume	-0.030	0.774	0.375	0.002

Figure 5 Relation between changes (Δ, follow-up minus baseline) in CRP and changes in total plaque volume, total plaque burden and necrotic core (NC) volume in patients presenting with stable angina pectoris (SAP, open bullet points) and acute coronary syndrome (ACS, closed bullet points)



	SAP		ACS	
	$r_{spearman}$	P-value	$r_{spearman}$	P-value
$LCBI_{ROI}$	-0.057	0.653	-0.148	0.375
$LCBI_{worst\ 10mm}$	-0.024	0.849	-0.150	0.369
$LCBI_{worst\ 4mm}$	0.049	0.699	-0.190	0.253

Figure 6 Relation between baseline CRP and baseline lipid core burden index (LCBI) in the total region of interest ($LCBI_{ROI}$) and the 10 mm ($LCBI_{10mm}$) and 4 mm ($LCBI_{10mm}$) sections with worst values in patients presenting with stable angina pectoris (SAP, open bullet points) and acute coronary syndrome (ACS, closed bullet points)



	SAP		ACS	
	$r_{spearman}$	P-value	$r_{spearman}$	P-value
$\Delta LCBI_{ROI}$	0.063	0.620	0.087	0.602
$\Delta LCBI_{worst\ 10mm}$	0.142	0.262	-0.172	0.301
$\Delta LCBI_{worst\ 4mm}$	0.177	0.163	-0.207	0.212

Correlation with ΔCRP (mg/L)

Figure 7 Relation between changes (Δ, follow-up minus baseline) in CRP and changes in lipid core burden index (LCBI) in the total region of interest ($LCBI_{ROI}$) and the 10 mm ($LCBI_{10mm}$) and 4 mm ($LCBI_{10mm}$) sections with worst values in patients presenting with stable angina pectoris (SAP, open bullet points) and acute coronary syndrome (ACS, closed bullet points)

ample, the median (IQR) delta $LCBI_{4mm}$ was only +10 points (IQR: -68, +103). We did not observe any association between changes in CRP levels and these key NIRS characteristics, neither in SAP, nor in ACS patients (Figure 7).

DISCUSSION

In our series of 164 patients with established CAD we found clinically relevant reductions in CRP levels after 1 year intensive (rosuva)statin therapy. These changes were observed in SAP as well as in ACS patients, but most prominent in ACS. Furthermore, in the ACS patients that we studied, CRP changes were correlated with changes in IVUS-derived plaque characteristics. We found no such correlations in SAP patients. CRP changes were uncorrelated with the also observed reductions in LDL-C.

Our findings are in agreement with previous studies that demonstrated that intensive statin treatment is capable to reduce serum cholesterol (LDL-C) as well as CRP levels (7,13,14). Our study also concurs with observations in CAD patients that (reductions in) CRP and LDL-C levels by statin therapy are largely uncorrelated. For example, in a series of 3745 ACS patients who received atorvastatin (80 mg/day) or

pravastatin (40 mg/day) for a mean of 24 months, less than 3% of the variation in achieved CRP levels was explained by the variation in achieved LDL-C (13). Furthermore, in that study, the achieved CRP and LDL-C were independently associated with the incidence of adverse coronary events. The relevance of reducing inflammation to prevent coronary events in patients with established CAD, independent of reducing LDL-C, was recently demonstrated in the CANTOS (4,5) and COLCOT trials (12).

As reported earlier, we found no relation between changes in LDL-C and changes in IVUS-derived plaque characteristics after 1 year of intensive (rosuva)statin treatment (11). In ACS patients, we now observed that changes in CRP levels were positively correlated with changes in total plaque volume, plaque burden and NC burden. These observations confirm the findings of SATURN and IBIS-4 studies in patients with ST-elevation myocardial infarction (STEMI) (15,16). In particular, in SATURN, a 'nonincreasing' CRP level was associated with a regression in plaque burden, whereas on-treatment CRP and not LDL-C was associated with adverse coronary events. We found no relation between CRP changes and changes in IVUS-derived plaque characteristics in SAP patients. In order to explain this observation, one might speculate that SAP and ACS patients have been shown to exhibit differences in pro-inflammatory and oxidative state (17). Still, it contrasts the findings by the REVERSAL investigators, who found a significant relation between CRP changes and changes in total plaque volume after 18 months of statin therapy in 502 patients with angiographically confirmed CAD (18). (Besides, interestingly, the REVERSAL investigators also reported a positive correlation between changes in LDL-C and coronary plaque characteristics.) It also contrasts, to some extent, with our findings in ATHEROREMO-IVUS, that CRP was associated with plaque volume and plaque burden in a mixed population of SAP and ACS patients, with no heterogeneity between the two phenotypes (19). Furthermore, in ATHEROREMO-IVUS, in SAP patients correlations were observed between TNF-α (positive) and IL-10 (negative) and plaque burden (20). In view of these mixed results, it appears that the relation between LDL-C reduction, inhibition of (vascular) inflammation, changes in extent and composition of coronary plaques, and coronary event reduction is not straightforward. Single study insights might easily by influenced by the clinical phenotype, intensity of the (statin) treatment, treatment duration, sample size and imaging techniques. For example, the median baseline CRP levels of the patients in the studies that we reference ranged from 1.5 (SATURN) to 4.2 (CANTOS, JUPITER) mg/L (4,7,8). Our current findings with respect to NIRS-derived LCBI should be

interpreted in this context: we found no relation between CRP (changes) and LCBI (changes), whereas we have earlier reported on a strong association between LCBI and MACE events (21,22), and others emphasized the prognostic value of serum CRP levels (4,5).

Limitations

Some limitations of this study need to be acknowledged. First, our sample size (in particular the subpopulations of SAP and ACS patients) was small, so that clinically relevant relations might have been missed. Second, intravascular imaging was performed in one pre-specified target segment of a non-culprit vessel, based on the assumption that such segment would represent the patient's atherosclerotic disease in the larger coronary tree. Although this hypothesis is supported by the fact that intravascular imaging measures on this single segment contained prognostic value (21-23), it still may be debated. Third, at study entry, most of the patients already received high-dose statin therapy. Therefore, a maximal lowering of serum CRP levels might have already occurred. Thus, the additional or incremental effect of high dose rosuvastatin might be less evident in these patients. Fourth, IBIS-3 was designed as an uncontrolled, observational study. Hence, changes in the measured serum biomarkers and intracoronary imaging characteristics during 1 year follow-up cannot be causally linked with the high-intensity statin treatment, which all patients received.

Conclusion

After 1 year intensive rosuvastatin therapy clinically relevant reductions in CRP levels were observed in a series of patients with established CAD. The observed CRP changes were correlated with changes in IVUS-derived plaque characteristics in ACS patients, but not in SAP. CRP changes were uncorrelated with changes in LDL-C levels. Hence, our study supports the role of in inflammation CAD progression, but also emphasizes that the relation between LDL-C reduction, inhibition of (vascular) inflammation, changes in extent and composition of coronary plaques, and coronary event reduction is not straightforward.

REFERENCES

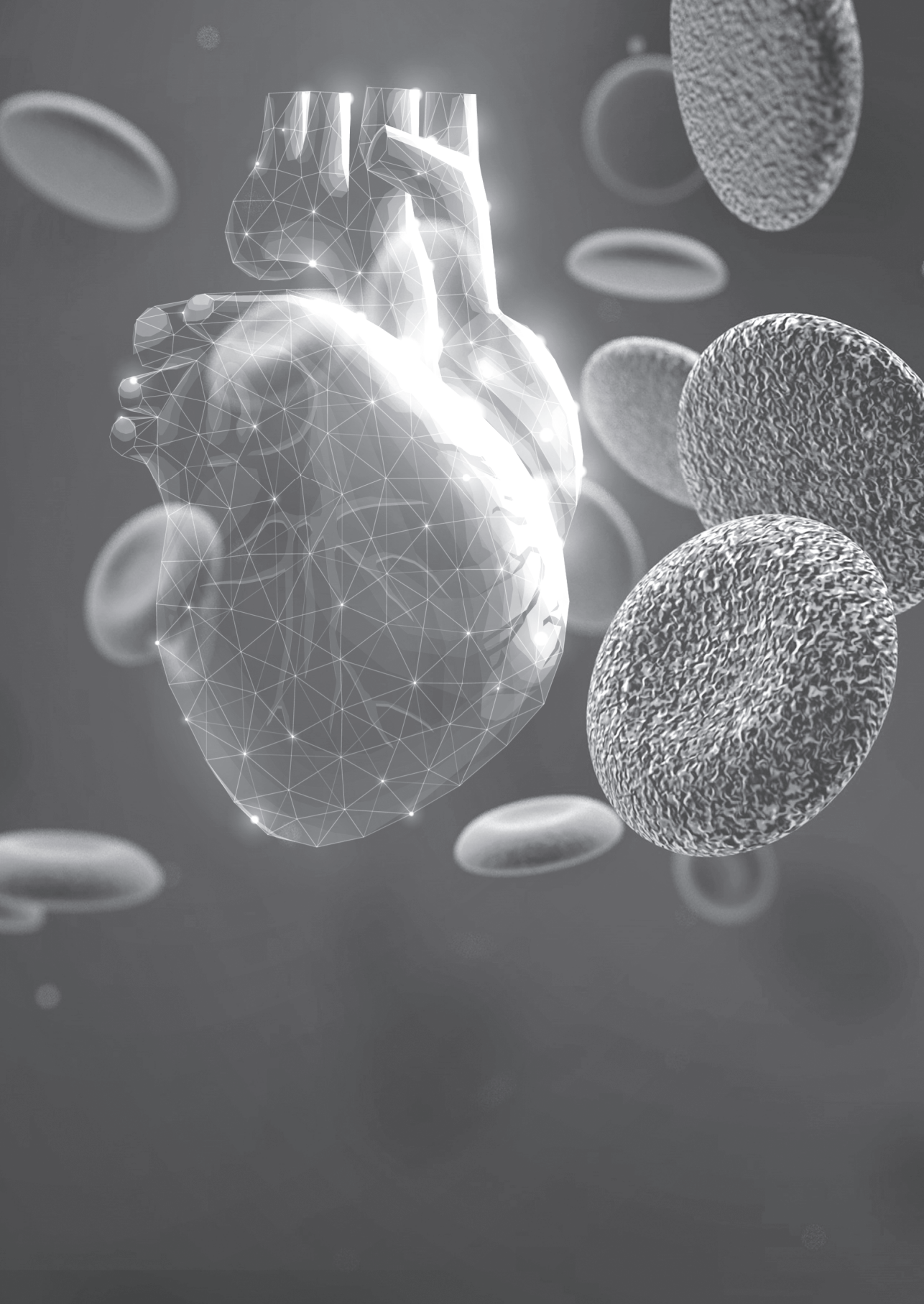
1. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002;105:1135-43.
2. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340:115-26.
3. Kaptoge S, Di Angelantonio E, Lowe G, Pepys MB, Thompson SG, Collins R, Danesh J. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis.Lancet. 2010; 375:132-40.
4. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ; CANTOS Trial Group. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease.N Engl J Med. 2017;377:1119-31.
5. Ridker PM, MacFadyen JG, Everett BM, Libby P, Thuren T, Glynn RJ, et al. Relationship of C-reactive protein reduction to cardiovascular event reduction following treatment with canakinumab: a secondary analysis from the CANTOS randomised controlled trial. Lancet. 2018;391:319-28.
6. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med. 2008;359:2195-207.
7. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, Macfadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ; JUPITER Trial Study Group. Reduction in C-reactive protein and LDL cholesterol and cardiovascular event rates after initiation of rosuvastatin: a prospective study of the JUPITER trial. Lancet. 2009;373:1175-82.
8. Puri R, Nissen SE, Libby P, Shao M, Ballantyne CM, Barter PJ, et al. C-reactive protein, but not low-density lipoprotein cholesterol levels, associate with coronary atheroma regression and cardiovascular events after maximally intensive statin therapy. Circulation. 2013;128(22):2395-403.
9. Nicholls SJ, Ballantyne CM, Barter PJ, Chapman MJ, Erbel RM, Libby P, et al. Effect of two intensive statin regimens on progression of coronary disease. N Engl J Med. 2011;365:2078-87.
10. Simsek C, Garcia-Garcia HM, van Geuns RJ, Magro M, Girasis C, van Mieghem N, et al. The ability of high dose rosuvastatin to improve plaque composition in non-intervened coronary arteries: rationale and design of the Integrated Biomarker and Imaging Study-3 (IBIS-3). EuroIntervention. 2012;8:235-41.
11. Oemrawsingh RM, Garcia-Garcia HM, van Geuns RJ, Lenzen MJ, Simsek C, de Boer SP, Van Mieghem NM, Regar E, de Jaegere PP, Akkerhuis KM, Ligthart JM, Zijlstra F, Serruys PW, Boersma E. Integrated Biomarker and Imaging Study 3 (IBIS-3) to assess the ability of rosuvastatin to decrease necrotic core in coronary arteries. EuroIntervention. 2016;12:734-9.
12. Tardif JC, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, Pinto FJ, Ibrahim R, Gamra H, Kiwan GS, Berry C, López-Sendón J, Ostadal P, Koenig W, Angoulvant D, Grégoire JC, Lavoie MA, Dubé MP, Rhainds D, Provencher M, Blondeau L, Orfanos A,

L'Allier PL, Guertin MC, Roubille F. Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. N Engl J Med. 2019;381:2497-505.

13. Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, Pfeffer MA, Braunwald E; Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 (PROVE IT-TIMI 22) Investigators. C-reactive protein levels and outcomes after statin therapy. N Engl J Med. 2005;352:20-8.
14. Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, Orazem J, Magorien RD, O'Shaughnessy C, Ganz P; Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) Investigators. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. N Engl J Med. 2005;352:29-38.
15. Puri R, Nissen SE, Libby P, Shao M, Ballantyne CM, Barter PJ, Chapman MJ, Erbel R, Raichlen JS, Uno K, Kataoka Y, Nicholls SJ. C-reactive protein, but not low-density lipoprotein cholesterol levels, associate with coronary atheroma regression and cardiovascular events after maximally intensive statin therapy. Circulation. 2013;128:2395-403.
16. Koskinas KC, Zaugg S, Yamaji K, García-García HM, Taniwaki M, Klingenberg R, Moschovitis A, Lüscher TF, van Tits LJ, Matter CM, Windecker S, Räber L. Changes of coronary plaque composition correlate with C-reactive protein levels in patients with ST-elevation myocardial infarction following high-intensity statin therapy. Atherosclerosis. 2016;247:154-60.
17. Lubrano V, Pingitore A, Traghella I, Storti S, Parri S, Berti S, Ndreu R, Andrenelli A, Palmieri C, Iervasi G, Mastorci F, Vassalle C. Emerging Biomarkers of Oxidative Stress in Acute and Stable Coronary Artery Disease: Levels and Determinants. Antioxidants (Basel). 2019;8. pii: E115. doi: 10.3390/antiox8050115.
18. Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, Orazem J, Magorien RD, O'Shaughnessy C, Ganz P; Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) Investigators. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. N Engl J Med. 2005;352:29-38.
19. Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Akkerhuis KM, Kardys I, de Boer SP, Langstraat JS, Regar E, van Geuns RJ, Serruys PW, Boersma E. 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;114:497-503.
20. Battes LC, Cheng JM, Oemrawsingh RM, Boersma E, Garcia-Garcia HM, de Boer SP, Buljubasic N, Mieghem NA, Regar E, Geuns RJ0, 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;236:18-24.
21. Oemrawsingh RM, Cheng JM, García-García HM, van Geuns RJ, de Boer SP, Simsek C, Kardys I, Lenzen MJ, van Domburg RT, Regar E, Serruys PW, Akkerhuis KM, Boersma E; ATHEROREMO-NIRS Investigators. Near-infrared spectroscopy predicts cardiovascular outcome in patients with coronary artery disease. J Am Coll Cardiol. 2014 Dec 16;64(23):2510-8.
22. Schuurman AS, Vroegindewey M, Kardys I, Oemrawsingh RM, Cheng JM, de Boer S, Garcia-Garcia HM, van Geuns RJ, Regar ES, Daemen J, van Mieghem NM, Serruys PW, Boersma E, Akkerhuis KM. Near-infrared spectroscopy-derived lipid core burden in-

dex predicts adverse cardiovascular outcome in patients with coronary artery disease during long-term follow-up. Eur Heart J. 2018;39:295-302.

23. Schuurman AS, Vroegindewey MM, Kardys I, Oemrawsingh RM, Garcia-Garcia HM, van Geuns RJ, Regar E, Van Mieghem NM, Ligthart J, Serruys PW, Boersma E, Akkerhuis KM. Prognostic Value of Intravascular Ultrasound in Patients With Coronary Artery Disease. J Am Coll Cardiol. 2018;72:2003-2011.



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E-Transmission of ECGs for expert consultation results in improved triage and treatment of acute ischemic chest pain patients by ambulance paramedics

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E-Transmission of ECGs for expert consultation results in improved triage and treatment of acute ischemic chest pain patients by ambulance paramedics

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ABSTRACT

Aims: In pre-hospital settings handled by paramedics, identification of myocardial infarction (MI) patients remains challenging when automated electrocardiogram ECG-interpretation is inconclusive. We aimed to identify those patients and to get them on the right track to (primary) percutaneous coronary intervention (PCI).

Methods and results: In the Rotterdam-Rijnmond region, automated ECG-devices on all ambulances were supplemented with a modem, enabling transmission of ECGs for on-line interpretation by an expert. The diagnostic protocol for acute chest pain was modified and monitored during 1 year.
Patients with an ECG that met the criteria for ST-segment elevation (STE) myocardial infarction (STEMI) were immediately transported to a PCI hospital. ECGs that did not meet the STEMI criteria, but showed total ST-deviation ≥800 μv were transmitted for on-line interpretation by the ECG-expert. On-line supervision was offered as a service in case ECGs showed conduction disorders, or had an otherwise 'suspicous' pattern according to the ambulance paramedics.

We enrolled 1076 acute ischemic chest pain patients who did not meet the automated STEMI criteria. Their mean age was 63 years; 64% were men. After on-line consultation, 735 (68%) patients were directly transported to a PCI-hospital for further treatment. PCI within 90 minutes was performed in 115 patients with a final MI diagnosis.

Conclusion: During a 1-year evaluation of the modified pre-hospital triage protocol for acute ischemic chest pain patients, over 100 MI patients with an initially inconclusive ECG received primary PCI within 90 minutes. Because of these results, we decided to continue the operation of the modified protocol.

INTRODUCTION

In patients presenting with acute chest pain suggestive of ongoing myocardial infarction (MI) early diagnosis and revascularization treatment leads to favourable clinical outcomes. Patients with ST-segment elevation (STE) myocardial infarction (STEMI) benefit most from percutaneous coronary intervention (PCI) when performed within 2 hours after symptom onset.(1-3) In The Netherlands, early mortality was reported to be as low as 1.6% in patients who receive PCI treatment in the first hour after symptom onset, compared with 4.0% in those treated after 5 hours. (4) Similarly low mortality has been reported after early PCI in non-ST-segment-elevation acute coronary syndrome (NSTE-ACS) patients with a so-called 'high-risk profile', including patients with a GRACE risk score >140.(5, 6) Thus, minimizing total ischemic time is the key to improve the prognosis of STEMI and high-risk NSTE-ACS patients, which mainly is a logistical challenge that starts in the pre-hospital setting.

For decades, the standard 12-lead electrocardiogram (ECG) has been the main diagnostic tool in the assessment of patients with acute chest pain. Worldwide, in most patients presenting with symptoms suggestive of ongoing MI in a pre-hospital setting an ECG will be obtained by the emergency medical service (EMS). Ambulances in The Netherlands, which are staffed by paramedics, are equipped with a patient-monitoring device that is capable to not only derive and register such ECGs, but to also provide an automated analysis and interpretation. Patients with an ECG that is interpreted as 'evolving MI' are then directly transported with highest emergency for coronary angiography and revascularization therapy to the nearest hospital with PCI service. Patients with an inconclusive ECG are transported to non-PCI hospitals for further diagnosis and treatment.

Satisfying results have been reported in relation to the implementation of ECG-based triage protocols,(3) also in the Rotterdam-Rijnmond region, The Netherlands, albeit in the thrombolysis era.(7) Still, in pre-hospital settings, it remains challenging to adequately identify those patients who require immediate reperfusion therapy when automated ECG analysis provides inconclusive results. In the past years, we have obtained anecdotic reports that acute ischemic chest pain patients who initially were transported to a non-PCI center in our region turned out to need immediate PCI after all. Review of their medical records showed that the automated ECG interpretation fell short to recognize the ongoing MI, and, consequently, symptom-onset-to-reperfusion times exceeded the guideline-recommended treatment criteria.

Because of these reports, in December 2013 we decided to change the logistic system in our region. The automated ECG-devices on the ambulances were then supplemented with a modem, which enabled e-transmission of the ECGs for expert consultation. We modified the diagnostic protocol, utilizing this technical option, and we hypothesized that a substantial portion of MI patients would get on the right track to (primary) PCI faster. The implementation of the new protocol was monitored during a one year period, and this paper presents the main findings.

METHODS

Setting

At the start of this study (in 2013), the Rotterdam-Rijnmond region in the Western part of The Netherlands holds a population of 1.1 million. A total of 10 hospitals are located in the region, two of which (*Erasmus MC* and *Maasstadziekenhuis*) offer a 24/7 primary PCI service for MI patients. Most acute ischemic chest pain patients have their first medical contact with a paramedic of the ambulance crew – it should be noted that, in the Netherlands, similar to most other (European) countries, a medical doctor is not present on the ambulance. The paramedic performs a brief physical examination and provides an initial diagnosis, which is mainly based on an automated analysis of the ECG. All ambulances in the region are equipped with the Corpuls3 defibrillator-/monitoringsystem, in which Biosigna HES PRO ECG-interpretation software (algorithm Rev.2.2) was implemented. Patients with a confirmed evolving MI are then transported to a PCI-hospital, whereas the remaining patients are transported to the nearest non-PCI-hospital.

Automated ECG interpretation

In November 2013, the Corpuls devices on the ambulances in the Rotterdam-Rijnmond region were enriched with modems which enabled transmission of the ECGs for on-line interpretation by an ECG-expert: the on-call cardiologist or cardiology resident in one of the two PCI-hospitals (Erasmus MC and Maasstadziekenhuis). Since then, the ECG-protocol for pre-hospital MI diagnosis in acute ischemic chest pain patients (which is a prerequisite) is as follows (Figure 1):

The existing protocol remained unchanged for patients with an ECG that shows ST elevation ≥200 μv in ≥2 adjacent anterior leads, or ≥100 μv in ≥2 non-anterior leads (Category 2). They are directly transported to a PCI-hospital, as they meet the STEMI criteria and need immediate revascularization. An alert is sent by the

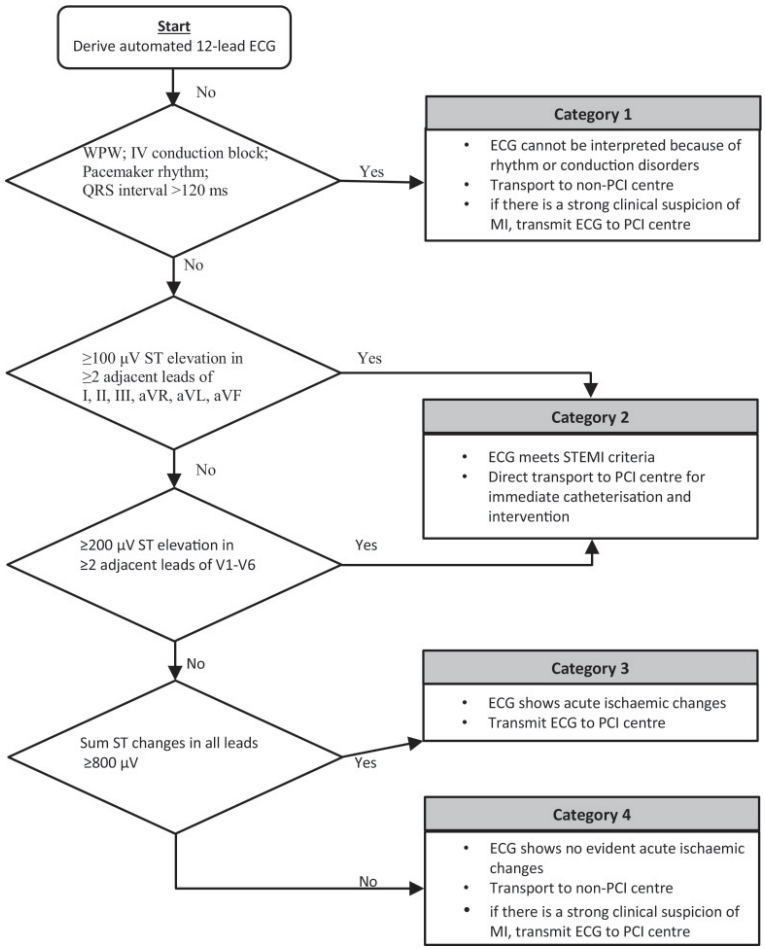


Figure 1. The ECG-protocol for pre-hospital myocardial infarction diagnosis

The following categories are distinguished by the automated ECG analysis and interpretation:

Category 1: ECG with rhythm or conduction disorders. Normally the patient is transported to a centre without facilities for PCI. If there is a strong clinical suspicion of evolving MI, the ECG will be transmitted for online interpretation by the ECG expert.

Category 2: ECG shows ST elevation ≥200 µv in ≥2 adjacent anterior leads, or ≥100 µv in ≥2 non-anterior leads. The ECG meet the criteria for STEMI. Immediate revascularisation is indicated, and patients are directly transported to a PCI hospital. The ambulance staff sends an alert to the PCI hospital, and transmits the ECG for completion of the medical dossier.

Category 3: ECGs that do not meet the STEMI criteria, but still show total ST deviation ≥800 µv must now be transmitted for online interpretation by the ECG expert.

Category 4: Abnormal ECGs without evident acute ischaemic changes.

As in Category 1, the patient is transported to a non-PCI centre. If there is a strong clinical suspicion of evolving MI, the ECG will be transmitted for online interpretation by the ECG expert (ECG electrocardiogram, IV intraventricular, MI myocardial infarction, PCI percutaneous coronary intervention, STEMI ST-elevation myocardial infarction, WPW Wolff-Parkinson-White)

ambulance staff to the PCI-hospital, and the ECG is transmitted for completion of the medical record.

With respect to the treatment of other patients, the existing protocol was extended. ECGs that do not meet the STEMI criteria, but still show total ST-deviation ≥800 μv (Category 3) must now be transmitted for on-line interpretation by the ECG-expert. According to the advice of the ECG-expert, which is provided by telephone within 5 minutes, the patient is then transported to the on-call PCI-hospital for immediate angiography, possibly followed by revascularization, or to a non-PCI-hospital for further evaluation by a cardiologist. For patients with an ECG that cannot be interpreted by the ECG-interpretation software because of conduction disorders (Category 1), as well as for patients with abnormal ECGs, but not showing evident acute ischemic changes (Category 4), on-line supervision by the ECG-expert is offered as a service that is not obliged.

Study patients and data collection

We monitored the revised protocol during the 1 year period from December 2013 to November 2014. Transmitted ECGs and primary data were collected by the ambulance personnel, including age, sex, ECG transmission date and time, and the diagnostic classification that was generated by the Corpuls device. Secondary data were collected by the first author (SA), based on a review of hospital medical records, and included medical history, risk factors, reperfusion time, final (discharge) diagnosis, and other pertinent clinical outcomes. All data were recorded in a dedicated database.

The patients who did not fullfill the STEMI criteria on the ambulance ECG were the population of main interest (Categories 1, 3, and 4). Still, we also collected information on patients who met the STEMI criteria (Category 2), and whose ECGs were transmitted.

Study endpoints

The revised protocol was developed to increase the early rule-in of MIs, and to increase the number of patients undergoing 'primary' PCI within the recommended 90 minutes window (PCI_{90min}), in particular in those patients who initially did not fullfill the STEMI criteria. PCI_{90min} was therefore defined as the main study endpoint. PCI delay was defined as the time difference between the acquirement of the ECG (time zero) and the wire crossing. The revised protocol also aimed to avoid unnecessary patient burden, invasive diagnostics (coronary catheterization) and treatments.

From this perspective, we considered patients that were immediately transported to a PCI-center, but who did not undergo PCI during the initial hospitalisation, as 'false positives'. Consequently, $PCI_{hospitalisation}$ was defined as the secondary endpoint. PCI_{90min} is an inappropriate endpoint in this respect, since it will be influenced by logistic delays.

We classified patients according to their final diagnosis as MI, Unstable Angina (UA), or 'other'. The diagnostic- and treatment criteria that were used by the treating physicians were based on prevailing European Society of Cardiology guidelines (8-10) This study was embedded in the clinical practice of the ambulance service and hospitals in the Rotterdam-Rijnmond region, and we accepted the final (hospital discharge) diagnosis that was made by the treating cardiologist. We derived this information from the hospital discharge letter, and we did not install an adjudication board to evaluate diagnoses and treatment decisions.

Statistical analysis

Baseline characteristics between the four diagnostic categories were compared. Continuous variables are presented as mean value ± standard deviation (SD) and categorical variables are presented as numbers and percentages.

PCI_{90min} and $PCI_{hospitalisation}$ are reported in relation to the diagnostic category. We conducted logistic regression analyses to examine the relation between diagnostic category and patient characteristics as predictor variables, and $PCI_{hospitalisation}$ as outcome (Category 2 patients are excluded from this analysis). Results are presented as unadjusted and adjusted odds ratios (OR) with 95 % confidence intervals (CI). These analyses might be useful to identify patient categories in which the diagnostic system was apparently and definitely - as by judgment of the treating physician - unsuccessful.

Data were analyzed with SPSS software (SPSS 23.0 IBM corp., Armonk, NY, USA). Statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Ethics

This is an observational study. For the purpose of this study, patients were not subject to acts, or imposed to any mode of behavior, other than standard treatment. For that reason, according to the Dutch law, written informed consent for a patient to be enrolled in this study was not necessary. This study was conducted according

Table 1 - Baseline characteristics according to the automated ECG-based initial diagnosis

	Automated ECG-based initial diagnosis					
	All patients	Category 1	Category 2	Category 3	Category 4	P-value
		ECG cannot be interpreted because of rhythm disturbances	ECG meets STEMI criteria	ECG shows acute ischemic changes	ECG shows no evident acute ischemic changes	
No. of patients	1421	228	345	526	322	
Demographic characteristics						
Age, years	62 ± 17	68 ± 14	57 ± 17	62 ± 18	61 ± 15	<0.001
Men	67	72	76	61	64	<0.001
Cardiovascular risk factors†						
Hypertension	53	64	37	58	60	<0.001
Hypercholesterolemia	41	47	28	43	48	<0.001
Diabetes mellitus	20	25	11	23	22	<0.001
Current smoker	34	27	47	33	40	<0.001
Positive family history	36	28	42	33	40	0.021
Cardiovascular history†						
CAD	31	43	17	32	38	<0.001
MI	22	30	14	19	31	<0.001
PCI	20	22	13	17	29	<0.001
CABG	7	11	1	9	8	<0.001
AF	10	17	2	12	9	<0.001

Data represent mean ± standard deviation values or percentages

† Data on cardiovascular risk factors and cardiovascular history were only available for the 1022 (72%) patients who were directly transported to PCI center. Data on smoking was complete in 89% and data on family history of coronary disease in 87% of patients.

Category 1: ECG with rhythm or conduction disorders. Category 2: ECG that shows ST elevation ≥200 μv in ≥2 adjacent anterior leads, or ≥100 μv in ≥2 non-anterior leads. Category 3: ECG that show total ST-deviation ≥800 μv. Category 4: abnormal ECG, without evident acute ischemic changes.

AF, atrial fibrillation; CABG, Coronary artery bypass grafting; CAD, coronary artery disease; ECG, electrocardiogram; MI, myocardial infarction; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction.

to the Privacy Policy of the Erasmus MC, and according to the Erasmus MC regulations for the appropriate use of data in patient-oriented research.

Table 2 - Final diagnosis and treatment according to the automated ECG-based initial diagnosis

	All patients	Category 1 ECG cannot be interpreted because of rhythm disturbances	Category 2 ECG meets STEMI criteria	Category 3 ECG shows acute ischemic changes	Category 4 ECG shows no evident acute ischemic changes	P-value
ECG transmitted to expert	1421	228	345	526	322	
Direct transport to PCI center after expert supervison	1022 (72)	182 (80)	287 (83)	333 (63)	220 (68)	<0.001
Final diagnosis						<0.001
Acute MI	431 (42)	76 (42)	222 (77)	85 (26)	48 (22)	
NSTEMI/UAP	144 (14)	31 (17)	12 (4)	69 (21)	32 (15)	
Other	447 (44)	75 (41)	53 (19)	179 (54)	140 (64)	
PCI performed in Acute MI*						
<90min	263/385 (68)	42/68 (62)	148/202 (73)	49/70 (70)	24/45 (53)	0.007
<120min	300/385 (78)	52/68 (76)	165/202 (82)	53/70 (76)	30/45 (67)	0.013
During hospitalization	400 (93)	71 (93)	211 (95)	72 (85)	46 (96)	0.073
PCI performed in STEMI/UAP						
<90min	14/86 (16)	1/17 (6)	2/8 (25)	8/44 (18)	3/17 (18)	0.827
<120min	16/86 (19)	2/17 (12)	2/8 (25)	9/44 (20)	3/17 (18)	0.775
During hospitalization	86 (60)	17 (55)	8 (67)	44 (64)	17 (53)	0.632

Data represent numbers (percentages)

* PCI during hospitalisation was performed in 400 of 431 patients with a final diagnosis of Myocardial Infarction. The other 46 (11%) patients had no indication for PCI due to medical conditions or other circumstances such as age, multivessel disease, preferred for CABG treatment based on occlusion of multiple cardiac blood vessels or other medical history. Note that data on the timing of the PCI was available in 385 patients.

Category 1: ECG with rhythm or conduction disorders.

Category 2: ECG that shows ST elevation ≥200 μv in ≥2 adjacent anterior leads, or ≥100 μv in ≥2 non-anterior leads.

Category 3: ECG that show total ST-deviation ≥800 μv.

Category 4: abnormal ECG, without evident acute ischemic changes.

Coronary Artery Bypass Grafting, CABG; ECG, electrocardiogram; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction.

RESULTS

Patient characteristics

The study cohort comprised 1421 patients with a mean age of 62 ± 17 years, and 67% were men (Table 1). A total of 345 patients met the STEMI criteria (Category 2 patients). As compared with the other categories (1,3,4) patients from category 2 were younger (mean age 57 versus 61-68 years), whereas the percentage men was higher (76 versus 61-72%). Furthermore, these patients had a somewhat more favorable cardiovascular disease risk profile, as fewer patients had hypertension

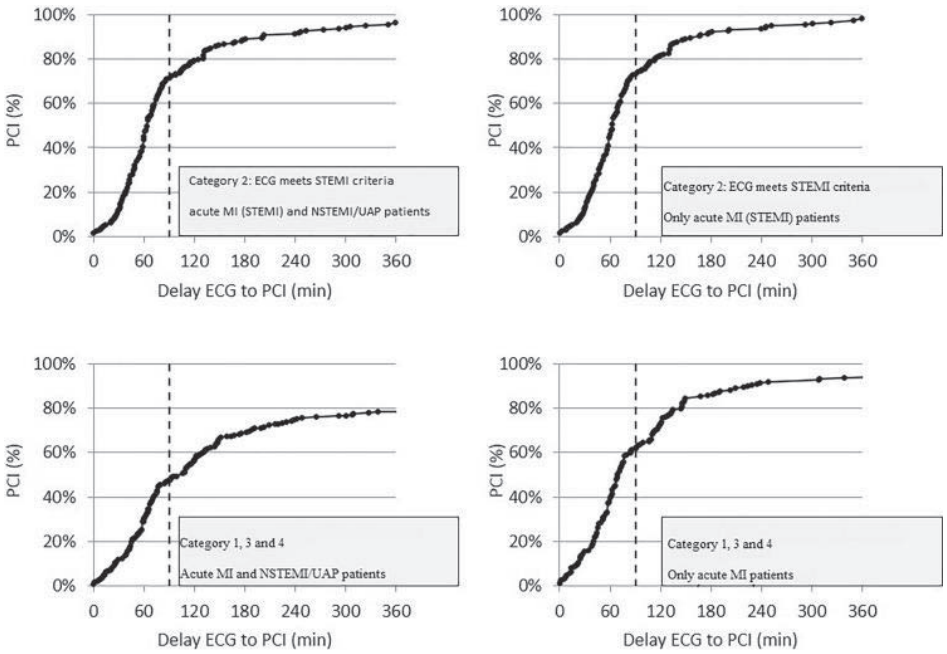


Figure 2. Time delay between ECG transmission and Percutaneous Coronary Intervention treatment

The results are presented as PCI (percentages) time delay (per minute) between the ECG transmission time and PCI treatment time for patients in Category 2 and the Categories 1, 3 and 4 combined.

Category 1: ECG with rhythm or conduction disorders. Category 2: ECG that shows ST elevation ≥200 μv in ≥2 adjacent anterior leads, or ≥100 μv in ≥2 non-anterior leads. Category 3: ECG that shows total ST deviation ≥800 μv. Category 4: abnormal ECG, without evident acute ischaemic changes

(ECG electrocardiogram, MI myocardial infarction, PCI percutaneous coronary intervention, NSTEMI non-ST-elevation myocardial infarction, STEMI ST-elevation myocardial infarction, UAP unstable angina pectoris)

(37 versus 58-64%), hypercholesterolemia (29 versus 43-47%), diabetes mellitus (11 versus 22-25%) and a history of coronary artery disease (17 versus 32-43%).

Initial diagnostic category and treatment decisions

As Table 2 demonstrates, a total of 287 (83%) of Category 2 patients were directly transported to a PCI-hospital. The reasons why the remaining 17% stayed home or were transported to a non-PCI hospital were not recorded. The final diagnosis was MI in 222 (77%) and UA in 12 (4%). Other diagnoses included pericarditis, costomyalgia and cardiomyopathy. $PCI_{hospitalisation}$ was performed in 211 (95%) cases with a

final MI diagnosis, whereas 73% had PCI_{90min}. A total of 8 (67%) patients with a final UA diagnosis had $PCI_{hospitalisation}$, and 25% had PCI_{90min}.

After on-line consultation with the ECG-expert, 735 (68%) Category 1, 3 and 4 patients were directly transported to a PCI-hospital for catheterization and further treatment. Final diagnosis was MI in 209 (28%) and UA in 132 (18%) patients. An indication for PCI was present in 189 of the MI patients. The remaining patients had (relative) contraindication for PCI because of advanced age, or had an indication for Coronary Artery Bypass Grafting (CABG) treatment.. The percentage of patients with a final diagnosis MI ranged from 22% in Category 4 to 42% in Category 1, whereas, in these MI patients, PCI_{90min} ranged from 53% (Category 4) to 70% (Category 3). Figure 2 shows details of time delays between ECG transmission and PCI treatment. Apparently, delays were longer for patients with an initial ECG that did not meet the STEMI criteria.

Determinants of $PCI_{hospitalisation}$ treatment

Table 3 shows determinants of $PCI_{hospitalisation}$. Patients presenting with an initial ECG that shows rhythm or conduction disturbances, which for that reason could not be analysed by the ECG-interpretation software, had considerably higher odds to receive $PCI_{hospitalisation}$ than those with abnormal, but interpretable ECGs (49 versus 30%, $OR_{adjusted}$ 2.7). Interestingly, patients with a history of atrial fibrillation had apparently lower odds for $PCI_{hospitalisation}$ than patients with normal rhythm (20 versus 41%, $OR_{adjusted}$ 0.25). Women had lower odds than men (31 versus 42%, $OR_{adjusted}$ 0.56), whereas elderly patients had higher odds ($OR_{adjusted}$ 1.04 per year). Also smoking status and a positive family history of coronary artery disease (CAD) appeared to be related to $PCI_{hospitalisation}$ treatment.

DISCUSSION

During a 1-year evaluation of the modified pre-hospital triage protocol for acute ischemic chest pain patients in the Rotterdam-Rijnmond region, 115 MI patients with an initially inconclusive ECG received primary PCI within 90 minutes, whereas another 20 received PCI within 90-120 minutes. Because of these results, we have decided to continue the operation of the modified protocol.

We initiated our project because we obtained anecdotical reports of acute ischemic chest pain patients in our region with an initially inconclusive ECG, who were transported to a non-PCI center, and who ultimately underwent immediate PCI for

MI. We intentionally designed an implementation study, and not a randomized trial, neither an observational before-after study. Accordingly, we cannot conclude with entire certainty that the observed early treatment was the direct consequence of a change in patient flow that was induced by the new triage protocol. Still, however, it must be appreciated that the original protocol recommended that these patients be transferred to a regional non-PCI hospital for further evaluation, whereas Miedema et al. observed that inter-hospital transfer was the most frequent cause of treatment delay in STEMI patients.(11) Wang et al. demonstrated in the Acute Coronary Treatment and Intervention Outcomes Network (ACTION) registry that door-in-door-out times from non-PCI- to PCI-hospitals might be as long as 68 minutes in 50% of patients.(12) Prior studies showed that <10% of STEMI patients with inter-hospital transfer were treated within 90 minutes and only 15% to 36% within 120 minutes.(13) These data support the benefits of the modified prehospital triage protocol in our region.

Several studies support the use of pre-hospital ECGs to reduce ischemic times in patients presenting with STEMI or NSTE-ACS.(14-16) Health care systems that involve trained paramedics for ECG interpretation,(17, 18) as well as systems that implemented automated ECG interpretation (19, 20) had satisfactory diagnostic performance and ditto beneficial results. Nevertheless, it has been demonstrated that a cardiologist or medical doctor overview and confirmation improves diagnostic accuracy,(21) while treatment delays are not increased.(22) In particular, ECG artifacts will then be avoided.(23) Our observation of an improved sensitivity to diagnose acute MI by the combination of automated ECG-interpretation and expert-consultation is in agreement with these studies.

It is equally important to filter out normal ECGs to avoid unnecessary treatments, and overcrowding at the PCI hospital.(24, 25) In our study, 64% of the Category 1, 3 and 4 patients that were immediately transported to a PCI center after on-line supervision by the ECG-expert did not undergo revascularisation during hospitalisation. Apparently, CAD requiring immediate treatment, and thus evolving MI, was excluded by the treating physician. In view of the observed benefits, we consider the 36/64 ratio acceptable, although there is room for improvement. Adding diagnostic- and risk-stratification tools could be helpful in this respect. We found that $PCI_{hospitalisation}$ was less likely in women, in younger patients, in non-smokers and in those without a family history of CAD. Still, differences were not large enough to justify a stratified approach according to these characteristics. The application of established risk stratifications scores in the pre-hospital setting, such as the Throm-

bolysis in Myocardial infarction (TIMI) risk score, Global Registry of Acute Coronary Events risk score (GRACE), or the history, ECG, age, risk factor and troponin (HEART) score might be beneficial to improve the diagnostic system.(26-28) Finally, research is warranted to evaluate the diagnostic performance of the combination of automated ECG-interpretation and out-of-hospital point-of-care troponin tests, which recently have become available.(29, 30)

Limitations

As by design, our study has several limitations that need to be mentioned. First, the telephone conversation between the ambulance-paramedic and the on-call ECG-expert was neither protocolized nor reported. As a result, we could not evaluate the factors that actually affected the reason for acceptance or refusal for immediate transportation to the PCI center. This is particular relevant for patients living in the zip code area of the PCI capable hospital. Most likely, the threshold to undergo early CAG in these patients is lower than for their counterparts living at further distance. Second, it is possible that the phone call, in which the clinical condition was discussed, had led to the admission, and not the transmitted and reviewed ECG per se. Unfortunately, however, we are not able to disentangle the influence of both phenomenon. Third, we did not follow-up the patients who stayed at home, or who were transported to a non-PCI center. We appreciate that patients who ultimately had MI might still have been missed in the prehospital phase, but, consequently, we cannot quantify their number. This is particularly the case for patients that were labelled by the ECG-interpretation software as Category 1 or 4. Fourth, we did not measure clinical outcomes. Thus - apart from the fact that our study is not a randomised trial - we are not able to demonstrate the benefit of the revised diagnostic system in terms of patient outcomes.

Conclusion

In conclusion, by applying the modified pre-hospital triage protocol for acute ischemic chest pain patients in the Rotterdam-Rijnmond region, during a 1-year period, over 100 MI patients with an initially inconclusive ECG received primary PCI within 90 minutes. The ‘false positive’ rate of 64% is considered acceptable. Still, further research is warranted to improve the specificity of the triage protocol, so that unnecessary burden to the patient and the system will be avoided.

REFERENCES

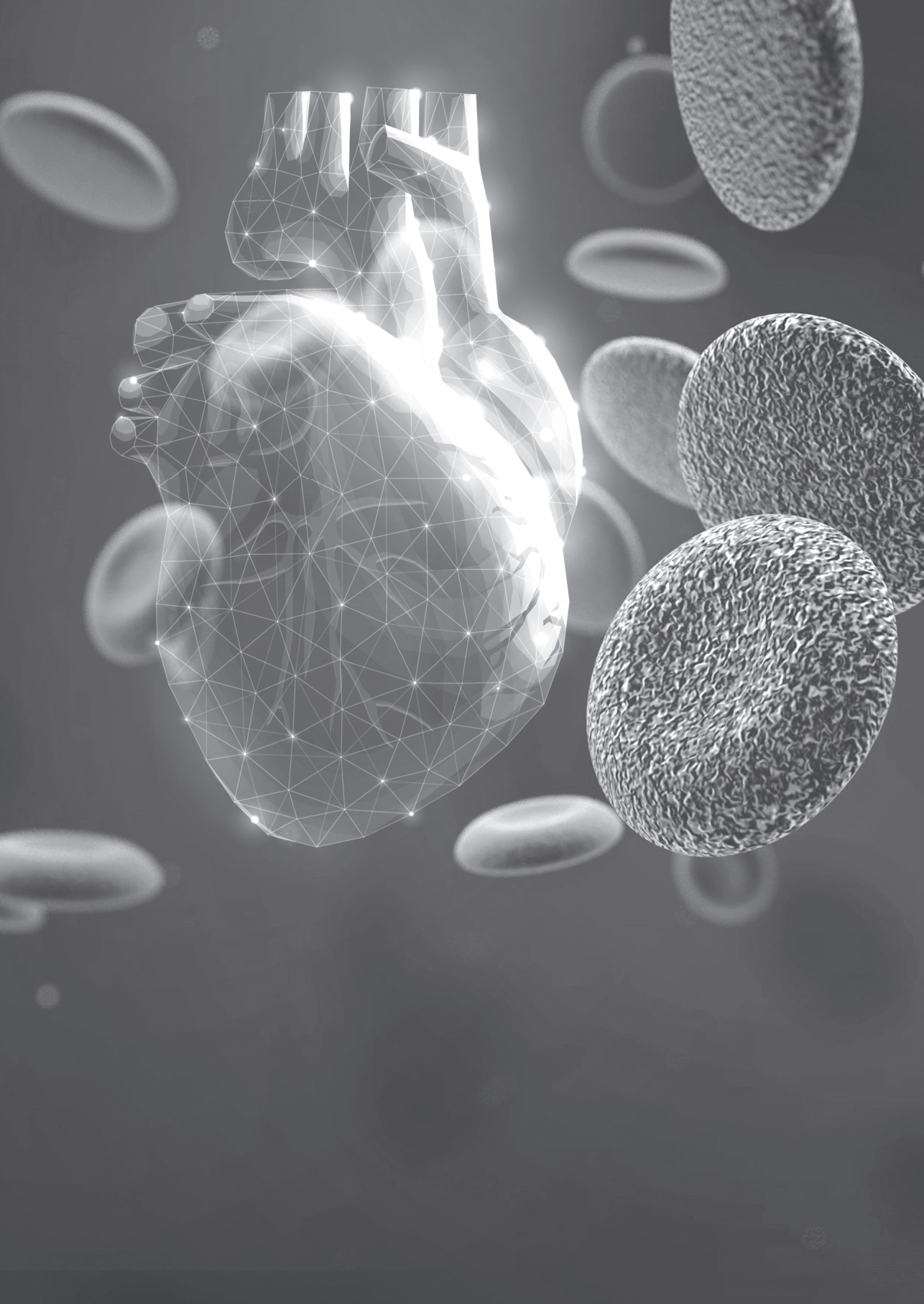
1. Terkelsen C. J., Sorensen J. T., Maeng M., et al. System delay and mortality among patients with STEMI treated with primary percutaneous coronary intervention. JAMA. 2010;304(7):763-71.
2. Rathore S. S., Curtis J. P., Chen J., et al. Association of door-to-balloon time and mortality in patients admitted to hospital with ST elevation myocardial infarction: national cohort study. BMJ. 2009;338:b1807.
3. Carstensen S., Nelson G. C., Hansen P. S., et al. Field triage to primary angioplasty combined with emergency department bypass reduces treatment delays and is associated with improved outcome. Eur Heart J. 2007;28(19):2313-9.
4. Fokkema M. L., Wieringa W. G., van der Horst I. C., Boersma E., Zijlstra F., de Smet B. J. Quantitative analysis of the impact of total ischemic time on myocardial perfusion and clinical outcome in patients with ST-elevation myocardial infarction. Am J Cardiol. 2011;108(11):1536-41.
5. Jobs A., Mehta S. R., Montalescot G., et al. Optimal timing of an invasive strategy in patients with non-ST-elevation acute coronary syndrome: a meta-analysis of randomised trials. Lancet. 2017;390(10096):737-46.
6. Milosevic A., Vasiljevic-Pokrajcic Z., Milasinovic D., et al. Immediate Versus Delayed Invasive Intervention for Non-STEMI Patients: The RIDDLE-NSTEMI Study. JACC Cardiovasc Interv. 2016;9(6):541-9.
7. Boersma E., Maas A. C., Hartman J. A., Ilmer B., Vos J., Simoons M. L. [Twelve year triage and thrombolysis treatment prior to hospitalization for myocardial infarction patients in the Rotterdam area of the Netherlands: outstanding short-term and long-term results] 12 jaar triage en trombolytische behandeling voor ziekenhuisopname bij hartinfarctpatienten in de regio Rotterdam: uitstekende korte- en langetermijnresultaten. Ned Tijdschr Geneeskd. 2001;145(42):2029-35.
8. Ibanez B., James S., Agewall S., et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). European heart journal. 2017.
9. Roffi M., Patrono C., Collet J. P., et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). Eur Heart J. 2016;37(3):267-315.
10. Steg P. G., James S. K., Atar D., et al. ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. European heart journal. 2012;33(20):2569-619.
11. Miedema M. D., Newell M. C., Duval S., et al. Causes of delay and associated mortality in patients transferred with ST-segment-elevation myocardial infarction. Circulation. 2011;124(15):1636-44.
12. Wang T. Y., Nallamothu B. K., Krumholz H. M., et al. Association of door-in to door-out time with reperfusion delays and outcomes among patients transferred for primary percutaneous coronary intervention. JAMA. 2011;305(24):2540-7.

13. Chakrabarti A., Krumholz H. M., Wang Y., Rumsfeld J. S., Nallamothu B. K., National Cardiovascular Data Registry. Time-to-reperfusion in patients undergoing interhospital transfer for primary percutaneous coronary intervention in the U.S: an analysis of 2005 and 2006 data from the National Cardiovascular Data Registry. J Am Coll Cardiol. 2008;51(25):2442-3.
14. Quinn T., Johnsen S., Gale C. P., et al. Effects of prehospital 12-lead ECG on processes of care and mortality in acute coronary syndrome: a linked cohort study from the Myocardial Ischaemia National Audit Project. Heart. 2014;100(12):944-50.
15. Cheskes S., Turner L., Foggett R., et al. Paramedic contact to balloon in less than 90 minutes: a successful strategy for st-segment elevation myocardial infarction bypass to primary percutaneous coronary intervention in a canadian emergency medical system. Prehosp Emerg Care. 2011;15(4):490-8.
16. Daudelin D. H., Sayah A. J., Kwong M., et al. Improving use of prehospital 12-lead ECG for early identification and treatment of acute coronary syndrome and ST-elevation myocardial infarction. Circ Cardiovasc Qual Outcomes. 2010;3(3):316-23.
17. O'Donnell D., Mancera M., Savory E., Christopher S., Schaffer J., Roumpf S. The availability of prior ECGs improves paramedic accuracy in recognizing ST-segment elevation myocardial infarction. J Electrocardiol. 2015;48(1):93-8.
18. Lee C. H., Van Gelder C. M., Cone D. C. Early cardiac catheterization laboratory activation by paramedics for patients with ST-segment elevation myocardial infarction on prehospital 12-lead electrocardiograms. Prehosp Emerg Care. 2010;14(2):153-8.
19. Potter B. J., Matteau A., Mansour S., et al. Sustained Performance of a "Physicianless" System of Automated Prehospital STEMI Diagnosis and Catheterization Laboratory Activation. Can J Cardiol. 2017;33(1):148-54.
20. Bradley E. H., Herrin J., Wang Y., et al. Strategies for reducing the door-to-balloon time in acute myocardial infarction. N Engl J Med. 2006;355(22):2308-20.
21. Huitema A. A., Zhu T., Alemayehu M., Lavi S. Diagnostic accuracy of ST-segment elevation myocardial infarction by various healthcare providers. Int J Cardiol. 2014;177(3):825-9.
22. Mawri S., Michaels A., Gibbs J., et al. The Comparison of Physician to Computer Interpreted Electrocardiograms on ST-elevation Myocardial Infarction Door-to-balloon Times. Crit Pathw Cardiol. 2016;15(1):22-5.
23. Bosson N., Sanko S., Stickney R. E., et al. Causes of Prehospital Misinterpretations of ST Elevation Myocardial Infarction. Prehosp Emerg Care. 2017;21(3):283-90.
24. Dieker H. J., Liem S. S., El Aidi H., et al. Pre-hospital triage for primary angioplasty: direct referral to the intervention center versus interhospital transport. JACC Cardiovasc Interv. 2010;3(7):705-11.
25. Larson D. M., Menssen K. M., Sharkey S. W., et al. "False-positive" cardiac catheterization laboratory activation among patients with suspected ST-segment elevation myocardial infarction. JAMA. 2007;298(23):2754-60.
26. Van Den Berg P., Body R. The HEART score for early rule out of acute coronary syndromes in the emergency department: a systematic review and meta-analysis. European heart journal Acute cardiovascular care. 2017:2048872617710788.
27. Antman E. M., Cohen M., Bernink P. J., et al. The TIMI risk score for unstable angina/non-ST elevation MI: A method for prognostication and therapeutic decision making. JAMA. 2000;284(7):835-42.

28. Granger C. B., Goldberg R. J., Dabbous O., et al. Predictors of hospital mortality in the global registry of acute coronary events. Arch Intern Med. 2003;163(19):2345-53.
29. Kip M. M. A., Koffijberg H., Moesker M. J., MJ I. Jzerman, Kusters R. The cost-utility of point-of-care troponin testing to diagnose acute coronary syndrome in primary care. BMC Cardiovasc Disord. 2017;17(1):213.
30. Van Hise C. B., Greenslade J. H., Parsonage W., Than M., Young J., Cullen L. External validation of heart-type fatty acid binding protein, high-sensitivity cardiac troponin, and electrocardiography as rule-out for acute myocardial infarction. Clin Biochem. 2017.

PART II

Blood biomarkers in heart failure



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In search of an efficient strategy to monitor disease status of chronic heart failure outpatients: added value of blood biomarkers to clinical assessment

8

In search of an efficient strategy to monitor disease status of chronic heart failure outpatients: added value of blood biomarkers to clinical assessment

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ABSTRACT

Background: Blood biomarkers carry potential for monitoring severity of chronic heart failure (CHF). Studies correlating repeated measurements of blood biomarkers with repeatedly assessed NYHA class over a prolonged follow-up period, and concomitantly investigating their associations with clinical endpoints, $_{have}$ not yet been performed.

Methods: In 2011-2013, 263 CHF patients were included. At inclusion and subsequently every 3 months, we measured N-terminal pro-B-type natriuretic (NT-proBNP), high-sensitivity troponin T (Hs-TnT) and C-reactive protein (CRP), and assessed NYHA class. The primary endpoint comprised heart failure hospitalization, cardiovascular mortality, cardiac transplantation or left ventricular assist device implantation. Time-dependent Cox models were used.

Results Mean age was 67±13 years, 72% were men and 27% were in NYHA class III–IV. We obtained 886 repeated measures (median 3[IQR 2-5] per patient). The primary endpoint was reached in 41 patients during a median follow-up of 1.0[0.6-1.4] year. Repeatedly measured NT-proBNP and Hs-TnT were significantly associated with repeatedly assessed NYHA class, whereas CRP was not (NT-proBNP: β[95%CI]:1.56[1.17–2.06]ln(ng/L) increase per point NYHA class, p=0.002; HsTNT: β[95%CI]:1.58[1.21–2.07]. Serially measured NT-proBNP (HR[95%CI]:1.37[1.18–1.60], CRP (1.17[1.06–1.29]) and NYHA class (1.29[1.13–1.47]) were positively and independently associated with the primary endpoint, whereas Hs-TnT lost statistical significance after multivariable adjustment. A model containing serially measured NYHA-class and NT-proBNP displayed a c-index of 0.84, while serially measured NYHA-class and CRP showed a c-index of 0.82.

Conclusions: Temporal NT-proBNP, CRP and NYHA class patterns are independently associated with adverse clinical outcome. Serially measured NT-proBNP and NYHA-class are best suited for monitoring CHF outpatients.

INTRODUCTION

Adjustment of medicinal treatment for chronic heart failure (CHF) requires considerable clinical acumen and may in some cases cause misjudgement in risk assessment and consequently suboptimal treatment.[1-4] Therefore, several diagnostic tools have been developed over the past decades which aim to objectify disease severity, such as the New York Heart Association (NYHA) Functional Classification,[1,4] which has limited reproducibility and high inter-observer variability.[5] Conversely, circulating blood biomarkers are less subjective to interpretation, and carry potential to monitor subtle changes in the heart that reflect and possibly predict adverse changes before they become clinically apparent.[6] The use of biomarkers, such as B-type natriuretic peptides (BNP), cardiac troponins and C- reactive protein (CRP), for risk stratification of CHF patients has already been demonstrated.[7-13] Moreover, although trials on natriuretic peptide-guided therapy of HF have provided somewhat inconsistent results,[9,14] natriuretic peptide-guided HF therapy has recently been given a class IIa recommendation in US HF guidelines to achieve guideline-directed medical therapy.[15,16]

Several studies have previously examined NYHA class in relation to clinical outcome in CHF patients. However, these studies either used single, baseline assessments or 2 repeated assessments taken in a relatively short time interval.[17,18] Furthermore, studies on the association between blood biomarkers and NYHA class in CHF are scarce, and studies assessing both these properties repeatedly are non-existent. Finally, the predictive value of serially assessed blood biomarkers and NYHA class scores for adverse clinical outcome has never yet been compared in stable CHF patients.

Therefore, the aim of the current investigation, performed in 263 patients with CHF, was to examine the associations between repeatedly measured NT-proBNP, troponin T (Hs-TnT), CRP, and NYHA class, as well as the associations of their temporal patterns with adverse clinical outcome. Based on this, we evaluated the incremental value of serially measuring blood biomarkers, to clinical assessment in terms of serial NYHA class scoring, for monitoring stable CHF outpatients.

METHODS

Patients

The *Serial biomarker measurements and new echocardiographic techniques in chronic heart failure patients result in tailored prediction of prognosis* (Bio-SHiFT) study was designed to investigate the hypothesis that temporal patterns of biomarkers involved in CHF are associated with prognosis. Bio-SHiFT is an ongoing prospective, observational study of stable outpatients with CHF, conducted in Erasmus MC, Rotterdam, The Netherlands and Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands. Patients were recruited during their regular outpatient visits and were in clinically stable condition. Patients were eligible for inclusion if aged 18 years or older, capable of understanding and signing informed consent, and if CHF (including HF with preserved ejection fraction) was diagnosed ≥3 months ago according to the guidelines of the European Society of Cardiology (ESC).[1,4,19] Detailed inclusion and exclusion criteria are shown in figure 1.

The study was approved by the medical ethics committees of the participating hospitals and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.The study is registered in ClinicalTrials.gov, number NCT01851538. Follow-up for this analysis lasted from October 2011 until November 2013.

Baseline assessment

At baseline, patients were evaluated by trained research physicians, who collected information on HF-related symptoms, including NYHA class.[1,4] History of chronic renal failure was defined as glomerular filtration rate less than 60 mL/min/1.73m^2. Alcohol consumption was defined as drinking ≥1 alcoholic consumption per day. Electrocardiography and echocardiography were performed. Data were entered into electronic case report forms. Non-fasting blood and urine samples were collected.

Follow-up visits

Study follow-up visits were scheduled every 3 months (a window of ±1 month was allowed), for a maximum of 30 months. At each tri-monthly study visit, a short medical evaluation was performed, NYHA functional class was scored and blood and urine samples were collected.

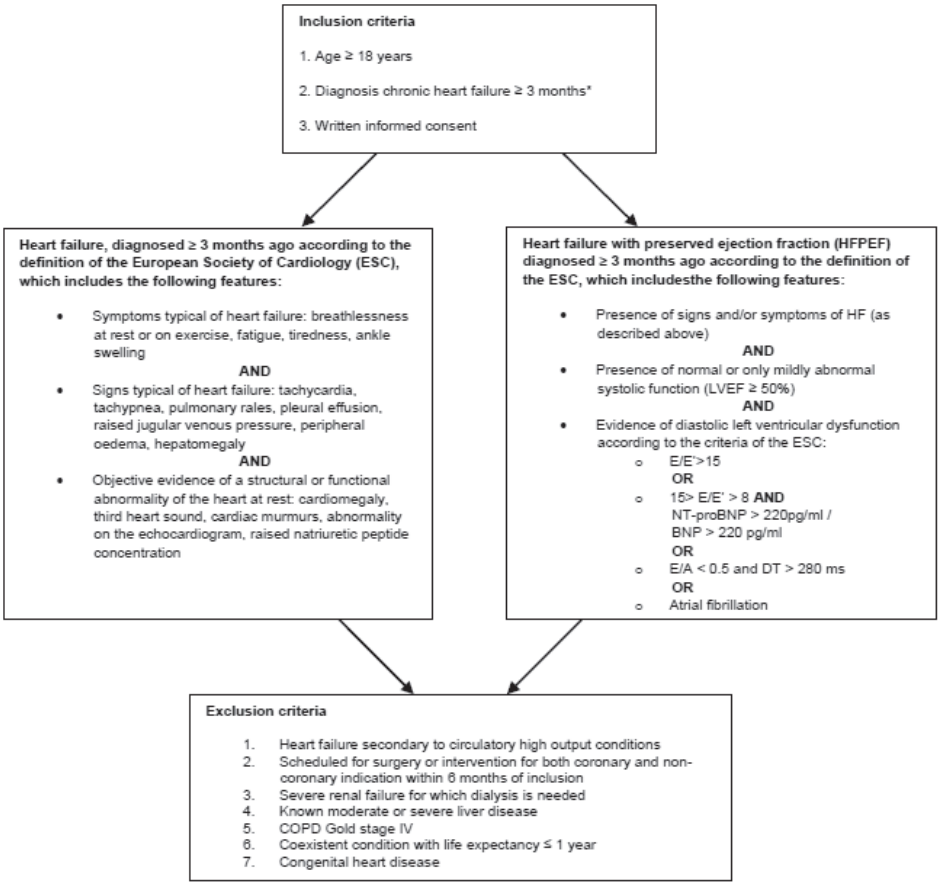


Figure 1. Inclusion and exclusion criteria

Blood sampling and biomarker measurement

Blood samples were collected at baseline and at each follow-up visit, and were processed and stored locally at a temperature of -80°C within 2 hours after blood collection. When applicable, samples were transported to the central laboratory (Erasmus MC, Rotterdam, The Netherlands) under controlled conditions (at a temperature of -80°C), until batch analysis was performed. Thus, the biomarker measurements performed for this study did not lead to treatment adjustments.

Batch analysis of NT-proBNP, Hs-TnT, and CRP was performed in the Clinical Chemistry Laboratory of the Erasmus MC. Plasma NT–proBNP and Hs-TnT were analysed using electrochemiluminesence immunoassays (Roche Diagnostics, Elecsys 2010, Indianapolis, Indiana, USA). For NT-proBNP, concentrations were measured ranging from 5 to 35.000 pmol/L. Coefficients of variation (CVs) were <5% at mean values

ranging from 5.19-274 pmol/L. For Hs-TnT, concentrations were measured ranging from 3-10000 ng/L. CVs were <5% at mean values ranging from 12.7-1819 ng/L. CRP was measured using an immunoturbidimetric assay (Roche Hitachi 912 chemistry analyser, Basel, Switzerland). This system measures concentrations ranging from 0.3 to 350 mg/L, and CVs were <5% at mean values ranging from 0.84-284 mg/L.

Clinical study endpoints

During follow-up, endpoints were recorded in the electronic case report forms by trained research physicians, and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee blinded to the biomarker results reviewed all collected information and adjudicated primary and secondary endpoints.

The primary endpoint comprised the composite of cardiac death, cardiac transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened HF.

Cardiac death was defined as death from myocardial infarction (MI) or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalisation for acute or worsened HF was defined as exacerbation of symptoms typical of HF, in combination with 2 of the following: BNP or NT-proBNP >3x the upper limit of normal, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral oedema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.

Statistical analysis

Statistical methods are described in detail in the supplemental text. In brief, we used linear mixed models to assess the associations between serial biomarker measurements and repeated assessment of NYHA functional class. Associations between serial measurements of biomarkers and NYHA class, and occurrence of the primary endpoint, were examined by entering the serial measurements as time-varying covariates into extended Cox proportional hazards models. First, the models were adjusted for age, gender, systolic blood pressure and estimated glomerular filtration rate (eGFR; calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation). Subsequently, all variables, i.e. NT-proBNP, Hs-TnT, CRP and NYHA functional class, were entered simultaneously into the models to

Table 1 – Baseline characteristics

	NYHA class I (n=78)	NYHA class II (n=116)	NYHA class III/IV (n=66)	Total (n=263)
Demographics				
Age	64 (±11)	68 (±13)	72 (±11)	67 (±13)
Male gender	60 (78)	81 (69)	48 (70)	189 (72)
Caucasian ethnicity	71 (92)	107 (92)	66 (94)	244 (94)
Clinical characteristics				
Body mass index kg/m^2	28 (±5)	27 (±4)	28 (±4)	28 (±5)
Heart rate, bpm	63 (±10)	68 (±11)	69 (±13)	67 (±12)
SBP, mmHg	123 (±19)	124 (±20)	120 (±21)	122 (±20)
DBP, mmHg	75 (±11)	72 (±11)	71 (±10)	72 (±11)
Biomarker level				
NT-proBNP (pmol/L)	93 (38-175)	141 (49-583)	225 (120-436)	140 (52-273)
HsTNT (ng/L)	13 (7.8-21)	19 (9.9-37)	24 (16-43)	18 (10 -33)
CRP (mg/L)	1.6 (0.6-3.4)	2.3 (1.0-5.3)	2.7 (1.3-4.9)	2.2 (0.9-4.8)
Creatinine (mg/dL)	1.2 (1.0 – 1.5)	1.1 (1.0 – 1.4)	1.3 (1.0 – 1.6)	1.2 (1.0 – 1.5)
eGFR[a] (ml/min/1.73 m2)	62 (42 – 83)	58 (46 – 78)	53 (38 – 72)	58 (43 – 76)
Medical history				
CAD	29 (39)	57 (50)	56 (84)	142 (46)
ICD	42 (55)	67 (57)	42 (61)	151 (59)
CRT	20 (26)	40 (34)	18 (26)	78 (30)
CVA	8 (10)	18 (15)	15 (22)	41 (16)
Chronic renal failure	34 (44)	61 (52)	41 (59)	136 (53)
Diabetes Mellitus	16 (23)	34 (28)	31 (42)	81 (31)
Hypercholesterolemia	26 (34)	41 (35)	26 (38)	93 (36)
Hypertension	33 (43)	56 (48)	31 (45)	120 (46)
Intoxications				
Alcohol consumption	30 (40)	51 (44)	27 (39)	108 (42)
Ever smoker	58 (75)	79 (68)	48 (70)	185 (71)
Medication use				
ACE-i or ARB	75 (96)	109 (94)	61 (88)	245 (93)
Aldosteron antagonist	44 (56)	81 (70)	53 (80)	178 (68)
Diuretic	64 (82)	107 (92)	66 (96)	237 (90)
Beta-blocker	68 (87)	106 (91)	58 (88)	232 (88)
Aspirin	14 (18)	16 (14)	15 (22)	45 (17)
Vitamin K antagonist	59 (77)	88 (78)	53 (77)	200 (77)

Normally distributed continuous variables are presented as mean (± standard deviation). Non-normally distributed continuous variables are expressed as median (25th – 75th percentile). Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts, because of missing values. ACE-i = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; CAD = coronary artery disease; CRP = C-reactive protein; CRT = cardiac resynchronisation therapy; CVA = cerebro vascular accident DBP = diastolic blood pressure; eGFR = estimated glomerular filtration rate; HsTNT = high sensitive cardiac troponin T; ICD = implantable cardioverter defibrillator; NT-proBNP = N-terminal pro-B-type natriuretic peptide; NYHA = New York Heart Association; SBP = systolic blood pressure.

[a]eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation

Table 2 – Associations between blood biomarker measurements, NYHA class, and the primary endpoint

Associations between baseline measurements and the primary endpoint				
	Univariable models[a]		Multivariable models[b]	
	HR (95% CI)	p	HR (95% CI)	p
NT-proBNP[c]	1.41 (1.22 – 1.63)	<0.001	1.26 (1.08 – 1.47)	0.003
Hs-TnT[c]	1.23 (1.10 – 1.37)	<0.001	1.11 (0.98 – 1.27)	0.11
CRP[c]	1.15 (1.04 – 1.27)	0.005	1.14 (1.03 – 1.26)	0.013
NYHA class[d]	1.29 (1.13 – 1.47)	<0.001	1.22 (1,07 – 1.40)	0.003
Associations between serial measurements and the primary endpoint				
	Univariable models[a]		Multivariable model[b]	
	HR (95% CI)	p	HR (95% CI)	p
NT-proBNP[c]	1.51 (1.32 – 1.73)	<0.001	1.34 (1.16 – 1.55)	<0.001
Hs-TnT[c]	1.23 (1.11 – 1.36)	<0.001	1.06 (0.95 – 1.18)	0.32
CRP[c]	1.26 (1.14 – 1.38)	<0.001	1.18 (1.07 – 1.30)	0.001
NYHA class[d]	1.36 (1.21 – 1.53)	<0.001	1.25 (1.11 – 1.42)	<0.001

HR= hazard ratio; CRP = C-reactive protein; Hs-TnT = high-sensitive cardiac troponin T; NT-proBNP = N-terminal pro-B-type natriuretic peptide; NYHA = New York Heart Association.

[a] Including age and gender

[b] Including age, gender, systolic blood pressure and estimated glomerular filtration rate

[c] HR per standard deviation increase in log transformed level

[d] HR per 1-step increase

investigate their independence. We calculated time-dependent C-indices based on the extended Cox models. Analyses were performed with R Statistical Software and MedCalc.

RESULTS

Study population

A total of 263 were included from October 2011 to June 2013. Baseline characteristics are displayed in table 1. Mean age was 67 years (SD±13), 72% were men, and 73% were in New York heart association (NYHA) class I or II. Median duration of HF at inclusion was 4.6 years (IQR 1.7–9.9). Median baseline NT-proBNP was 137.3 pmol/L (IQR 51.9–272.9), Hs-TnT:18.0 ng/L (IQR 9.6–33.2) and CRP:2.2 mg/L (IQR 0.9–4.8).

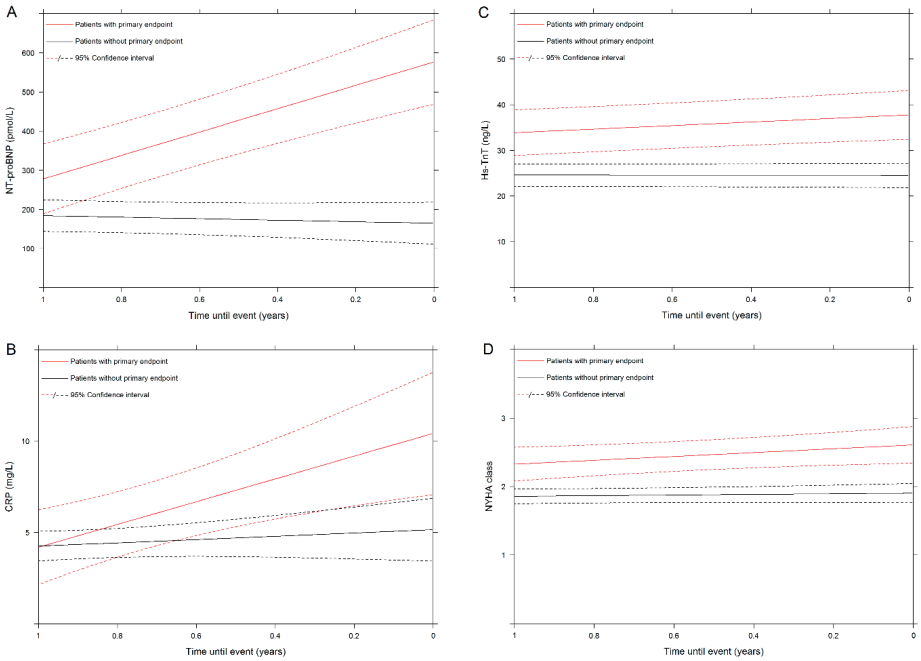


Figure 2. Temporal evolution of serial biomarkers measurements and NYHA class
X-axes display the time that is left until occurrence of the clinical endpoint.

Associations between serial biomarker measurements and serial assessment of NYHA class

During follow-up, we collected 921 blood samples and scored NYHA functional class 1292 times. Of all follow-up visits, 1135 took place before the occurrence of the primary endpoint. During these follow-up visits, 886 blood samples were drawn (median 3; IQR 2-5 per patient), and NYHA functional class was scored 1114 times (median 4; IQR 2-6 per patient).

Repeatedly measured NT-proBNP and Hs-TnT showed strong associations with repeatedly assessed NYHA class, whereas CRP did not (NT-proBNP: β[95%CI]:1.56[1.17–2.06]ln(ng/L) increase per point NYHA class, p=0.002; HsTNT: β[95%CI]:1.58[1.21–2.07], p=0.001; CRP: β[95%CI]:1.22[0.98–1.53], p=0.076) (supplemental table 1).

Clinical endpoints

The composite endpoint was reached by 41 patients (16%), during a median follow-up of 1.0 [0.6-1.4] years: 5 patients died from a cardiovascular cause, 35 patients were re-hospitalized for worsened HF and 1 patient underwent heart transplanta-

tion. Of the 35 patients reaching the primary endpoint because of re-hospitalisation for HF, 16 patients died eventually during further follow-up, of whom 12 patients died from a cardiovascular cause. Overall all-cause mortality was 21 (8.0%).

Baseline biomarker measurements and NYHA class and the primary endpoint

NT-proBNP, Hs-TnT, CRP, and NYHA class all displayed strong and positive associations with the primary endpoint (table 2). After multivariable adjustment, NT-proBNP, CRP and NYHA class remained independently associated with the primary endpoint, while, Hs-TnT lost statistical significance. Of all other baseline characteristics, only age was independently associated with the primary endpoint.

Serial measurements of biomarkers and NYHA class, and the primary endpoint

Temporal evolutions of serial biomarker measurements and NYHA class are displayed in figure 2. Serially measured NT-proBNP, Hs-TnT, CRP, and NYHA class all displayed strong and positive associations with the primary endpoint (table 2). After multivariable adjustment for all serially measured variables, NT-proBNP, CRP and NYHA class remained significantly associated with the primary endpoint.

Model performance

The discriminative abilities of the models containing baseline measurements of NT-proBNP, Hs-TnT, CRP and NYHA class are shown in supplemental table 2, and those of serial measurements in table 3. All individual serially assessed c-indices, except for Hs-TnT, were numerically higher than corresponding baseline c-indices. Adding serial NT-proBNP to the model containing age, sex, systolic blood pressure and eGFR and NYHA class, provided a substantial increase in C-index from 0.76(CI 0.66–0.86) to 0.84(0.74 – 0.93), although this did not reach statistical significance (p=0.26). Adding serial CRP instead to this same multivariable model resulted in a C-index of 0.82(0.72 – 0.92), p=0.40. Adding both serial NT-proBNP and CRP to the multivariable model only resulted in a slight improvement compared to addition of NT-proBNP only: 0.85(CI 0.75–0.95), p=0.20.

DISCUSSION

In this prospective, observational cohort of CHF patients, repeatedly measured NT-proBNP and Hs-TnT were positively and significantly associated with repeatedly

Table 3 – Discriminative ability of models containing serial blood biomarker- and NYHA assessment

Model	C-index (CI)	P-value
Model[a]	0.62 (0.52 – 0.71)	NA
Model[a] + NT-proBNP	0.80 (0.70 – 0.90)	0.010[b]
Model[a] + Hs-TnT	0.72 (0.62 – 0.82)	0.13[b]
Model[a] + CRP	0.76 (0.66 – 0.85)	0.048[b]
Model[a] + NYHA class	0.76 (0.66 – 0.86)	0.040[b]
Model[a] + NYHA class + NT-proBNP	0.84 (0.74 – 0.93)	0.26[c]
Model[a] + NYHA class + Hs-TnT	0.79 (0.69 – 0.89)	0.67[c]
Model[a] + NYHA class + CRP	0.82 (0.72 – 0.92)	0.40[c]
Model[a] + NT-proBNP + CRP	0.82 (0.73 – 0.92)	0.40[c]
Model[a] + NYHA class + NT-proBNP + CRP	0.85 (0.75 – 0.95)	0.20[c]

CI = Confidence interval; CRP = C-reactive protein; Hs-TnT = High-sensitive cardiac troponin T; NT-proBNP = N-terminal pro-B-type natriuretic peptide; NYHA = New York Heart Association.

[a] Including age, gender, systolic blood pressure and estimated glomerular filtration rate

[b] P-value compared to model[a]

[c] P-value compared to model[a] + NYHA class

assessed NYHA class. Serial assessments of NT-proBNP, CRP and NYHA class were independently associated with adverse clinical outcome. Repeatedly measured NT-proBNP and CRP both added individually to serial NYHA-class assessments in terms of discriminative ability. However, a model combining both of these biomarkers with serially scored NYHA-class, seemed to have little incremental value over serial NYHA-class assessment combined with only one of these blood biomarkers. In particular, adding NT-proBNP only seemed the best suited strategy for monitoring stable CHF outpatients.

Strengths of the current study include simultaneous assessment of multiple biomarkers and NYHA class on the one hand, as well as frequent, repeated assessment of these properties on the other hand. Combined with clinical follow-up on adverse events, this renders insight into temporal evolution and manifestation of CHF. On top of that, using biomarker measurements for monitoring patients with CHF has the appealing feature of being objective, and thus uniform and reproducible.

Serial NT-proBNP and CRP measurements were both independently associated with the endpoint and adding serial NT-proBNP and CRP measurements to a model containing NYHA class assessments greatly increased the c-index, from 0.76 to 0.85. This increase did not reach statistical significance, but recently it has been demonstrated that testing for improvement in prediction performance is actually redundant if a variable has already been shown to be an independent risk factor,

and that standard testing procedures such as c-indices are very conservative and thus insensitive to improvements in prediction performance.[20] Nevertheless, to provide an impression of the magnitude of the incremental prognostic value, we presented C-indices. Altogether, our results support combining blood biomarkers with clinical assessment for prognostication in CHF patients.

While the prognostic value of blood biomarkers for clinical events has been widely investigated, less is currently known about the association between blood biomarkers and NYHA class in CHF patients. Only two studies have previously assessed this association. These studies measured natriuretic peptide level both at study baseline and at 6±2 weeks of follow-up, and correlated these measurements with, among others, clinical change as categorized by clinicians.[17,18] Studies performing multiple, repeated measurements of biomarkers and NYHA class over a prolonged follow-up period, and concomitantly investigating their association with clinical endpoints have not yet been performed. Although the NYHA functional classification is a common and globally used system,[1,4] its biggest disadvantage is the non-uniformity in its application by individual clinicians. Raphael et al conducted a study to investigate consistency in NYHA functional class assessment and found that inter-observer variability was high, with only 54% concordance between two cardiologists.[5] In this respect, adding biomarker information to clinical patient assessment could be valuable for obtaining a more objective estimate of patient prognosis.

Limitations

Extended Cox models with time-dependent covariates were used to analyse the effects of changes in NYHA class and temporal biomarker patterns on the primary endpoint, because these models are able to accommodate multiple time-varying covariates. However, time-dependent Cox models assume that biomarker levels do not change between measurements.[21] In reality, blood biomarkers are dynamic and continuously change over time, parallel to the condition of the patient. Therefore, we performed a sensitivity analysis by means of a joint modelling approach. Joint models combine a linear mixed-effects model for the serial biomarker measurements with a Cox proportional hazards model for the occurrence of the primary endpoint.[21] We estimated the individual biomarker trajectories and NYHA trajectories using separate joint models, then extracted the fitted trajectories from the joint models, and entered the extracted trajectories simultaneously into one extended Cox model. The results of this analysis were materially the same as those we described in the paper. Furthermore, the majority of the patient population was in

NYHA class I or II, and thus at relatively low risk. The results and conclusions should be judged accordingly.

Conclusion

Serial assessments of NT-proBNP and Hs-TnT are positively associated with NYHA class. Temporal patterns of NT-proBNP, CRP and NYHA class are independently associated with adverse clinical outcome. A model containing these serially measured variables displayed good discriminative ability. However, serially measured CRP had only little incremental discriminative value compared to a strategy combining serial assessments of NYHA class and NT-proBNP. Altogether, our findings underscore the incremental value of biomarkers to NYHA class for monitoring stable CHF outpatients.

REFERENCES

1. McMurray JJ, Adamopoulos S, Anker SD, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 2012;33:1787-1847.
2. Januzzi JL, Troughton R. Are serial BNP measurements useful in heart failure management? Serial natriuretic peptide measurements are useful in heart failure management. Circulation 2013;127:500-507.
3. Yancy CW, Jessup M, Bozkurt B, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. Circulation 2013;128:e240-e327.
4. Dickstein K, Cohen-Solal A, Filippatos G, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur Heart J 2008;29:2388-2442.
5. Raphael C, Briscoe C, Davies J, et al. Limitations of the New York Heart Association functional classification system and self-reported walking distances in chronic heart failure. Heart 2007;93:476-482.
6. de Couto G, Ouzounian M, and Liu PP. Early detection of myocardial dysfunction and heart failure. Nat Rev Cardiol 2010;7:334-344.
7. Felker GM, Hasselblad V, Hernandez AF, et al. Biomarker-guided therapy in chronic heart failure: a meta-analysis of randomized controlled trials. Am Heart J 2009;158:422-430.
8. Savarese G, Trimarco B, Dellegrottaglie S, et al. Natriuretic peptide-guided therapy in chronic heart failure: a meta-analysis of 2,686 patients in 12 randomized trials. PLoS One. 2013;8:e58287.
9. Porapakkham P, Porapakkham P, Zimmet H, et al. B-type natriuretic peptide-guided heart failure therapy: A meta-analysis. Arch Intern Med 2010;170:507-514.
10. Anand IS, Latini R, Florea VG, et al. C-reactive protein in heart failure: prognostic value and the effect of valsartan. Circulation 2005;112:1428-1434.
11. Miller WL, Hartman KA, Burritt MF, et al. Serial biomarker measurements in ambulatory patients with chronic heart failure: the importance of change over time. Circulation 2007;116:249-257.
12. Troughton RW, Frampton CM, Brunner-La Rocca HP, et al. Effect of B-type natriuretic peptide-guided treatment of chronic heart failure on total mortality and hospitalization: an individual patient meta-analysis. Eur Heart J 2014;35:1559-1567.
13. Yan RT, White M, Yan AT, et al. Usefulness of temporal changes in neurohormones as markers of ventricular remodeling and prognosis in patients with left ventricular systolic dysfunction and heart failure receiving either candesartan or enalapril or both. Am J Cardiol 2005;96:698-704.
14. Eurlings LW, van Pol PE, Kok WE, et al. Management of chronic heart failure guided by individual N-terminal pro-B-type natriuretic peptide targets: results of the PRIMA

(Can PRo-brain-natriuretic peptide guided therapy of chronic heart failure IMprove heart fAilure morbidity and mortality?) study. J Am Coll Cardiol 2010;56:2090-2100.
15. Troughton R, Michael FG, and Januzzi JL, Jr. Natriuretic peptide-guided heart failure management. Eur Heart J 2014;35:16-24.
16. Yancy CW, Jessup M, Bozkurt B, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 2013;62:e147-e239.
17. Luther SA, McCullough PA, Havranek EP, et al. The relationship between B-type natriuretic peptide and health status in patients with heart failure. J Card Fail. 2005;11:414-421.
18. Spertus J, Peterson E, Conard MW, et al. Monitoring clinical changes in patients with heart failure: a comparison of methods. Am Heart J 2005;150:707-715.
19. Paulus WJ, Tschope C, Sanderson JE, et al. How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. Eur Heart J 2007;28:2539-2550.
20. Pepe MS, Kerr KF, Longton G, et al. Testing for improvement in prediction model performance. Stat.Med. 2013;32:1467-1482.
21. Rizopoulos D, Takkenberg JJ. Tools & techniques--statistics: Dealing with time-varying covariates in survival analysis--joint models versus Cox models. Eurointervention 2014;10:285-288.

SUPPLEMENTAL TEXT

Statistical analysis

Variables with normal distributions are presented as mean±standard deviation (SD). Variables with non-normal distributions are presented as median and interquartile range (IQR). Categorical data are displayed as count and percentage. In case of skewed distributions, continuous variables were logarithmically transformed for further analyses.

To assess the associations between serial biomarker measurements and repeated assessment of NYHA functional class, we used linear mixed models. Time was used as a random effect. NYHA class was used as the independent variable (fixed effect), in order to be able to uniformly display the change in each of the biomarkers per point increase in NYHA class. Each of the biomarkers was consecutively used as the dependent variable. Associations amongst the 3 biomarkers were examined likewise. For these analyses, all samples drawn were used.

Associations between baseline values of NT-proBNP, Hs-TnT, CRP, and NYHA functional class on the one hand, and the primary endpoint on the other hand, were assessed using only the samples drawn before the occurrence of the primary endpoint. Cox proportional hazards models were used. Associations between serial measurements of the aforementioned variables and occurrence of the primary endpoint were examined by entering the serial measurements into extended Cox proportional hazards models as time-varying covariates. First, the models were adjusted for age, gender, systolic blood pressure and estimated glomerular filtration rate (eGFR; calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation). Subsequently, all variables, i.e. NT-proBNP, Hs-TnT, CRP and NYHA functional class, were entered simultaneously into the models to investigate their independence. For serial measurements, this meant that all variables were simultaneously entered as time-varying covariates into the extended Cox analysis. The multivariable models also included age, gender, systolic blood pressure and eGFR.

It has previously been demonstrated that testing for improvement in prediction performance is actually redundant if a variable has already been shown to be an independent risk factor. Independence already proves presence of incremental value [20]. Still, to provide an impression of the magnitude of the incremental discriminative ability of the individual and combined serial measurements of the biomarkers and NYHA functional class, we calculated time-dependent C-indices based on the extended Cox models.

Finally, to investigate the incremental value of serial biomarker measurements to baseline assessment only, we simultaneously added the baseline measurements and the series of longitudinal measurements of NT-proBNP, CRP, Hs-TnT and NYHA class into the models, in order to obtain separate hazard ratios for the baseline measurements and for the series of longitudinal measurements.

Analyses were performed with R Statistical Software using packages 'Survival' and 'nlme'. C-indices were compared using MedCalc. All tests were two-tailed and p-values <0.05 were considered statistically significant.

Supplemental table 1 – Associations between serial blood biomarker measurements and NYHA class

	NT-proBNP[a]					
NT-proBNP[a]	**B (95%CI)**	**p-value**	**Hs-TnT[a]**			
Hs-TnT[a]	1.66 (1.53 – 1.81)	<0.001	**B (95%CI)**	**p-value**	**CRP[a]**	
CRP[a]	1.11 (1.06 – 1.16)	<0.001	1.03 (0.99 – 1.08)	0.11	**B (95%CI)**	**p-value**
NYHA class[b]	1.56 (1.17 – 2.06)	0.002	1.58 (1.21 – 2.07)	0.001	1.22 (0.98 – 1.53)	0.076

CRP = C-reactive protein; Hs-TnT = high sensitive cardiac troponin T; NT-proBNP = N-terminal pro-B-type natriuretic peptide; NYHA = New York Heart Association.

[a] Beta coefficient per standard deviation increase in log transformed level

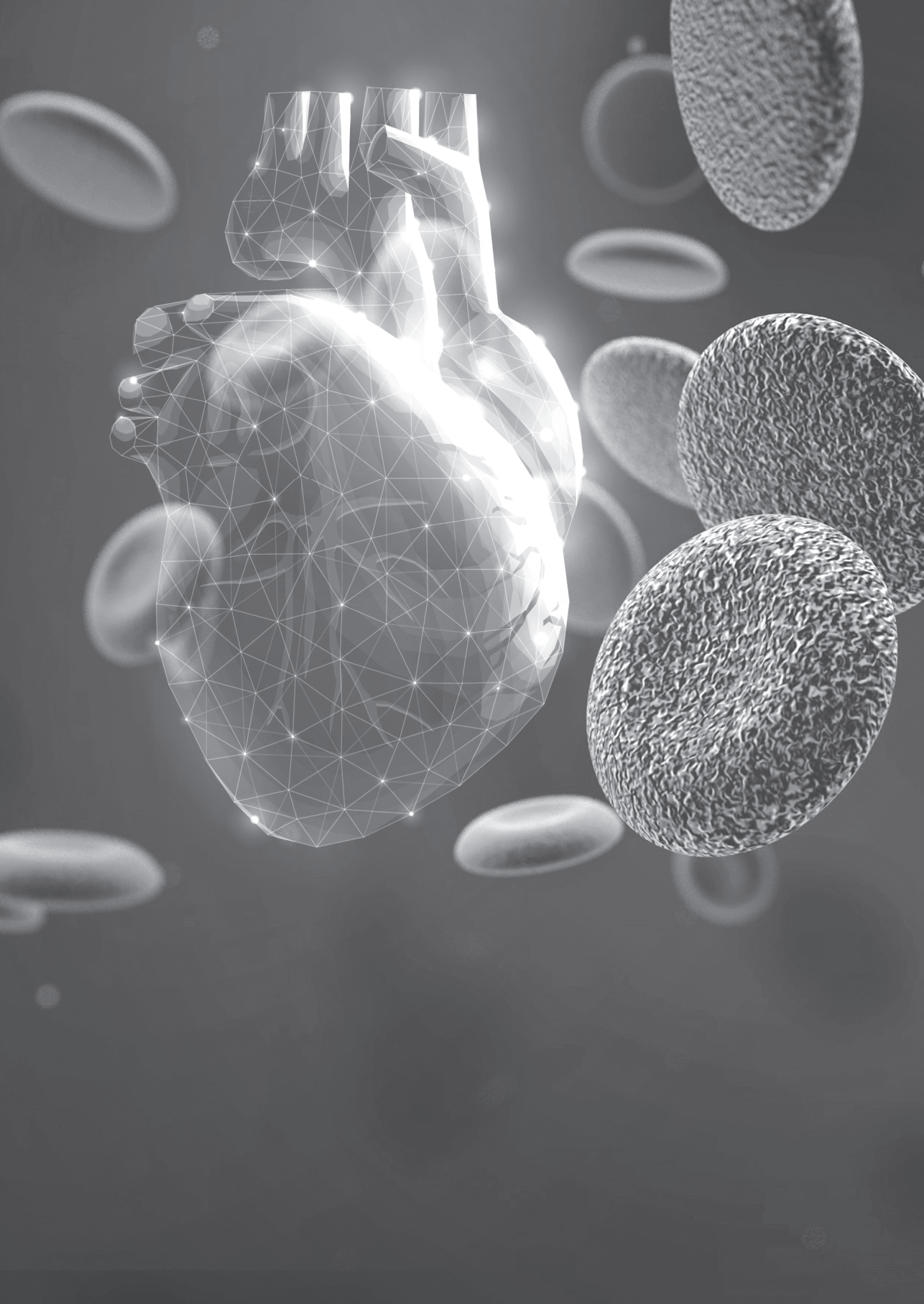
[b] Beta coefficient per 1-step increase

Supplemental table 2 – Discriminative ability of models containing baseline blood biomarker- and NYHA assessment

Model	C-index (CI)
Model[a]	0.62 (0.52 – 0.71)
Model[a] + NT-proBNP	0.76 (0.67 – 0.86)
Model[a] + Hs-TnT	0.72 (0.62 – 0.81)
Model[a] + CRP	0.68 (0.58 – 0.78)
Model[a] + NYHA class	0.71 (0.60 – 0.81)
Model[a] + NT-proBNP + CRP + Hs-TnT + NYHA class	0.80 (0.71 – 0.91)

CI = Confidence interval; CRP = C-reactive protein; Hs-TnT = High-sensitive cardiac troponin T; NT-proBNP = N-terminal pro-B-type natriuretic peptide; NYHA = New York Heart Association.

[a] Including age, gender, systolic blood pressure and estimated glomerular filtration rate.



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Toward personalized risk assessment in patients with chronic heart failure: Detailed temporal patterns of NT-proBNP, troponin T, and CRP in the Bio-SHiFT study

9

Toward personalized risk assessment in patients with chronic heart failure: Detailed temporal patterns of NT-proBNP, troponin T, and CRP in the Bio-SHiFT study

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ABSTRACT

Background: We examined the prognostic information of detailed temporal patterns of N-terminal pro B-type natriuretic peptide (NT-proBNP), high-sensitive troponin T (HsTNT) and C-reactive protein (CRP) in patients with chronic heart failure (CHF).

Methods: From 2011-2013, 263 CHF patients were included. NT-proBNP, HsTNT and CRP were measured at baseline and every 3 months. The primary endpoint (PE) comprised heart failure hospitalization, cardiovascular mortality, cardiac transplantation and LVAD-implantation. Associations between temporal biomarker patterns and the PE were investigated by joint modelling, which combines mixed models with Cox regression.

Results: Mean age was 67±12 years and 72% were men. Median follow-up was 2.2 (IQR 1.4–2.5) years. We used 2022 blood samples (median 9(IQR 5-10) per patient) and 70 (27%) patients reached the PE. Temporal patterns of NT-proBNP, HsTNT and CRP level were associated with the PE (multivariable adjusted HR per doubling of biomarker: NT-proBNP 2.28 (95%CI 1.82–2.86), HsTNT 2.05(1.63–2.58), CRP 1.65 (1.30–2.08). A combined 3 biomarker model demonstrated independent associations for the temporal patterns of NT-proBNP and CRP level (HRs 2.06(1.53–2.79) and 1.38(1.01–1.89), respectively). Instantaneous change in biomarker level was also independently associated with the PE for NT-proBNP and CRP. Long-term biomarker elevation showed an association for NT-proBNP.

Conclusions: Temporal patterns representing evolution of level and rate of change in level of NT-proBNP and CRP, and long-term elevation of NT-proBNP

are independently associated with adverse prognosis in CHF patients. Individual patterns of change and combining multiple biomarkers could carry value for prognostication and for therapy guidance.

INTRODUCTION

The diagnosis of progression of chronic heart failure (CHF) is primarily based on clinical signs and symptoms and the decision to adjust therapy is usually made once symptoms of progression have become manifest. Blood biomarkers are capable of monitoring subtle (patho)physiological processes that reflect and possibly predict adverse changes before they become clinically apparent. 1,2 B-type natriuretic peptides (BNP) and N-terminal proBNP (NT-proBNP), cardiac troponin T and I and C-reactive protein (CRP) have been unequivocally related to adverse clinical outcomes in heart failure (HF) patients in several large studies.2-12

The majority of these studies have examined single, baseline measurements of these blood biomarkers. However, since patients with CHF display large biological heterogeneity, distinguishing patients at different levels of risk of adverse events based on single biomarker measurements only is challenging. Measuring biomarkers repeatedly could contribute to individualized risk assessment. Studies that have assessed changing biomarker patterns over time have mostly focused on natriuretic peptides, generally used only few repeated biomarker measurements, and have utilized simplified representations of temporal biomarker evolution, such as change between two time-points.3-6,12-14 Results of these studies strongly depend on the statistical approach that was used.1 Subsequent trials on natriuretic peptide-guided therapy of HF have provided inconsistent results.8-10,15,16 Most of these trials did not use individualized target levels for natriuretic peptides, and did not take other biomarkers into consideration.

The above illustrates that in order to properly install personalized risk assessment that makes use of blood biomarkers, first, more detailed information is needed on temporal biomarker patterns in individual patients. Specifically, having measurements available that are performed closely in time to the moment that the endpoint of interest occurs, would provide further insight into the biomarkers' behaviour as this endpoint nearly approaches. This would enable an adequate investigation of whether, and to which degree, increasing (or decreasing) biomarker levels contribute to an individual's risk, regardless of whether his or her blood levels exceed classic, absolute cut-points at any random point in time (such as 'study baseline').

However, in practice, biomarker measurements performed shortly before the endpoint occurs are difficult to acquire, because they require a high frequency of blood sampling during prolonged follow-up. Therefore, most studies on this topic have performed only two measurements over time and are thus not able to properly investigate the biomarker trajectory shortly before the endpoint occurs.

In the current study, we have performed frequent (up to 11), repeated measurements of multiple blood biomarkers (NT-proBNP, HsTNT and CRP) in 263 patients with CHF, and have investigated the associations of the thus obtained temporal patterns with adverse clinical outcome. These 3 biomarkers were chosen because each of them represents different aspects of heart failure pathophysiology (wall stress, myocyte damage and inflammation) and because a large body of evidence exists for the prognostic value of single measurements of these markers. By performing multiple, longitudinal measurements, assessing multiple biomarkers simultaneously and using appropriate, modern statistical methods, we aimed to provide a basis for improved, personalized risk assessment in patients with CHF.

METHODS

Patients

Bio-SHiFT is a prospective, observational study of stable outpatients with CHF, conducted in Erasmus MC, Rotterdam, The Netherlands and Noordwest Ziekenhuisgroep, Alkmaar, The Netherlands. Patients were recruited during their regular outpatient visits and were in clinically stable condition. Detailed inclusion and exclusion criteria are shown in figure 1. Patients were eligible if CHF (including HF with preserved ejection fraction) was diagnosed ≥ 3 months ago according to the guidelines of the European Society of Cardiology (ESC).17-19 This study was approved by the medical ethics committee of the Erasmus MC, Rotterdam, The Netherlands and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. The study is registered in ClinicalTrials.gov, number NCT01851538. Estimated enrolment is 400 patients. In the current paper, we have performed an interim analysis on the 263 patients who were enrolled during the first inclusion period between October 2011 and June 2013.

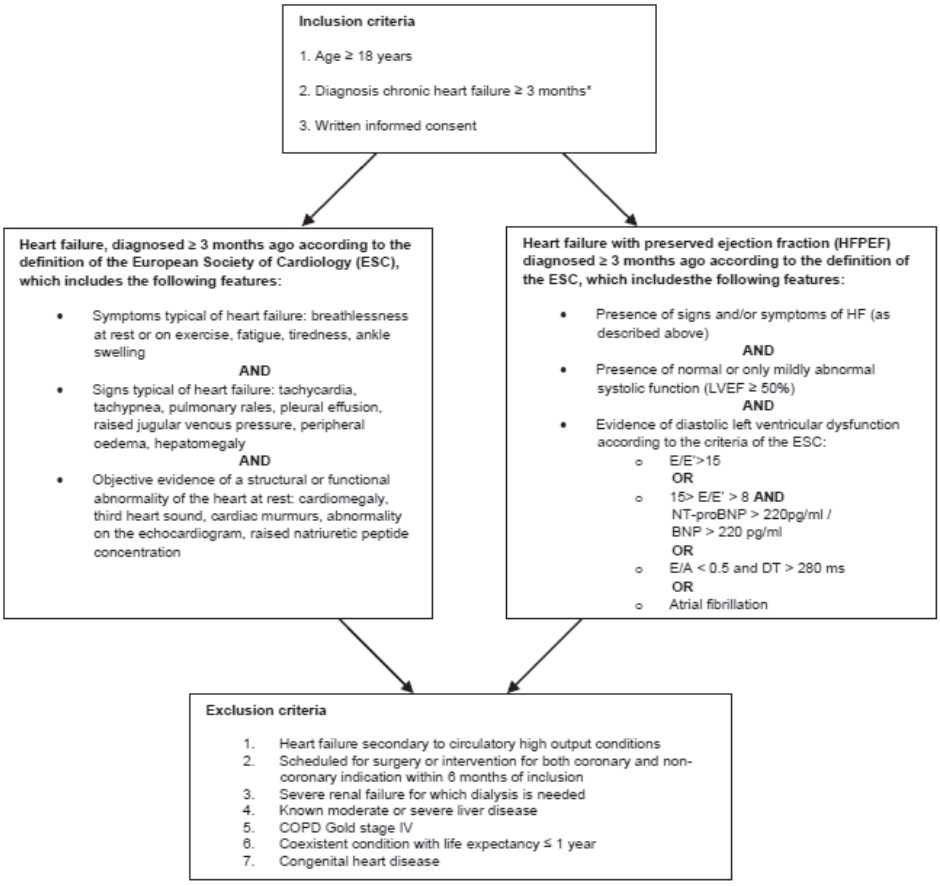


Figure 1 - Inclusion and exclusion criteria

Baseline assessment

At baseline, patients were evaluated by trained research physicians, who collected information on HF-related symptoms, including NYHA class,17,18 and performed physical examination, including blood pressure, heart rate and body mass index. Information on aetiology of heart failure, presence of systolic dysfunction, cardiovascular risk factors, medical history and medical treatment was retrieved primarily from hospital records. History of chronic renal failure was defined as glomerular filtration rate (GFR) less than 60 mL/min/1.73 m2. Alcohol consumption was defined as drinking ≥1 alcoholic consumption per day. Data were entered into electronic case report forms. Electrocardiography and echocardiography were performed. Non-fasting blood and urine were collected, as described below.

Follow-up visits

Routine outpatient follow-up by the treating physician continued for all patients during the study. Study follow-up visits were scheduled every 3 months (a window of ±1 month was allowed), to a maximum follow-up duration of 30 months. At each follow-up visit, a short medical evaluation was performed and blood and urine samples were collected. Changes in medication as well as occurrence of adverse cardiovascular events since the previous visit were recorded.

Blood sampling and biomarker measurement

Blood samples were processed and stored at a temperature of -80oC within 2 hours after blood collection. When applicable, samples were transported to the central laboratory (Erasmus MC, Rotterdam, The Netherlands) under controlled conditions (at a temperature of -80oC), until batch analysis was performed. Accordingly, results of the biomarker assays were not available to treating physicians at the time of the outpatient visits. Thus, the biomarker measurements performed for this study did not lead to drug adjustments, and all patients received usual care. This concurs with Bio-SHiFT being a strictly observational study, as described above.

For the purpose of the current analysis, three biomarkers (NT-proBNP, HsTNT, and CRP) were measured in one batch in stored serum samples. Plasma NT–proBNP was analysed using an electrochemiluminesence immunoassay (Roche Diagnostics, Elecsys 2010, Indianapolis, Indiana, USA), which measures concentrations ranging from 5 to 35.000 pmol/L. Cardiac troponin T was also measured using an electrochemiluminesence immunoassay (Roche Diagnostics, Elecsys 2010 immunoassay analyser, Indianapolis, Indiana, USA), measuring concentrations ranging from 3-10000 ng/L. CRP was measured using an immunoturbidimetric assay (Roche Hitachi 912 chemistry analyser, Basel, Switzerland). This system measures concentrations ranging from 0.3 to 350 mg/L. All coefficients of variation were <5%.

Clinical study endpoints

During follow-up, hospitalizations for HF, myocardial infarction (MI), percutaneous coronary interventions (PCIs), coronary artery bypass grafting (CABG), arrhythmias, and cerebrovascular accidents (CVAs), as well as cardiac transplantation, left ventricular assist device implantation (LVAD) and mortality, were recorded in the electronic case report form by trained research physicians, and associated hospital records and discharge letters were collected.

Subsequently, hospital records and discharge letters were reviewed by a clinical event committee blinded to the biomarker results, and primary and secondary endpoints were adjudicated. The primary endpoint comprised the composite of cardiac death, cardiac transplantation, LVAD- implantation, and hospitalization for HF, whichever occurred first in time. Secondary endpoints included individual components of the primary endpoint, and also MI, PCI, CABG, CVA, and all-cause mortality.

Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalisation for acute or worsened HF was defined as a hospitalisation for an exacerbation of HF symptoms, in combination with 2 of the following: BNP or NT-proBNP >3x ULN, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral oedema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.

Statistical analysis

Distributions of continuous variables, including biomarker concentrations, were tested for normality using the Kolmogorov-Smirnov test. Normally distributed continuous variables are presented as mean ± standard deviation (SD). Non-normally distributed continuous variables are expressed as median and interquartile range (IQR). Categorical data are displayed as count and percentage.

In case of skewed distributions, continuous variables were logarithmically transformed (log base 2) for further analyses. Associations between patient characteristics and baseline biomarker levels were evaluated using univariable linear regression. Associations between baseline patient characteristics, including baseline biomarker levels, and the primary endpoint, were evaluated using Cox proportional hazards models. These analyses were first performed univariably. Subsequently, to evaluate independent associations, all baseline characteristics that showed statistically significant associations (with p-values <0.05) were forced into a multivariable Cox model.

Associations between temporal biomarker patterns of each separate biomarker and the primary end point were assessed using a joint modeling approach, which combines a linear mixed-effects (longitudinal) sub model to assess the temporal evolution of the repeatedly measured marker with a Cox proportional-hazards

sub model to analyse the association of this temporal evolution with the study end point. In line with the logarithmic (base 2) transformation of the biomarker concentrations, the results are presented as hazard ratios (HRs) per doubling of the biomarker concentration at any point in time, along with the corresponding 95% CIs. First, analyses were performed univariably. Subsequently, potential confounders were entered into the joint models. These included all variables that were signifi- cantly associated with the primary end point in the multivariable "baseline" Cox proportional hazards model (NYHA class and diabetes mellitus), as well as variables selected from existing literature (age, gender, renal function, body mass index). Covariates were missing in less than 3% of patients. Multiple imputations (5 times) of these covariates were performed in the multivariable analyses.

The above-described analysis assesses the predictive value of repeatedly measured biomarker levels; specifically, it provides HRs that estimate the risk of the end point associated with doubling of biomarker level at any point in time. However, in the context of serial marker measurements, there could be additional features of the marker's trajectory that better predict the primary end point.20 Therefore, we investigated the predictive value of (1) the "instantaneous slope" of the marker's trajectory, indicating whether a marker is decreasing, is increasing, or remains stable, and (2) the area under the curve of the marker's trajectory, indicating the cumulative effect of all the values the marker has taken in the past (this area under the curve does not provide information on increasing or decreasing biomarker values, which should be derived from the slope).We chose not to correct for multiple testing, because the selection of the currently investigated 3 biomarkers was based on previous research and thus hypothesis-driven.2-12

To simultaneously investigate the effect of all 3 biomarkers on the primary end point and thus to assess their independent predictive value, all individual temporal biomarker patterns derived from the adjusted joint models were saved and subsequently entered simultaneously as time-varying covariates into an extended Cox analysis. The same approach was used to investigate the independent predictive value of the slope and the area under the curve of the 3 temporal biomarker patterns. Adjustment for potential baseline confounders was performed as described above. Additionally, these extended Cox models were adjusted for temporally changing total daily doses of equivalents of carvedilol, enalapril, furosemide, and spironolactone, which were also entered into the models as time-varying covariates.

To illustrate how joint modeling can be applied to estimate prognosis of an individual patient based on his or her repeatedly assessed biomarker values, we plotted the temporal patterns of the biomarkers in several individual patients (i.e., example patients drawn from our dataset) together with their corresponding dynamic, individual probabilities of survival as estimated by the joint model (which we developed on the total study population as described above). As such, we graphically demonstrated individual survival probabilities, which are updated each time that an additional measurement is performed in the patient as he or she visits the outpatient clinic.

Finally, to investigated the discriminative ability of models containing serial measurements and models containing baseline measurements only, we calculated c-indices based on extended Cox models containing temporal biomarker patterns derived from the adjusted joint models, as well as c-indices based on Cox models containing baseline biomarker values only.

All analyses were performed with R Statistical Software using package JM.20 All tests were two-tailed and p-values <0.05 were considered statistically significant.

Power calculation

The current investigation comprised 263 patients, of whom 70 reached the primary endpoint. For baseline measurements, these numbers are sufficient to detect odds ratios around 2 for the upper quintile of a biomarker associated with the endpoint (α-error 0.05, power of 80%) when comparing cases with non-cases. For repeated measurements, power is further enhanced. A median of 9 samples per patient were available. We calculated power for repeated measurements by assuming a linear association and a continuous autoregressive correlation matrix. We used NT-proBNP to derive the measurement error standard deviation (sigma, equal to 463) and the input parameter for the autoregressive correlation matrix (rho, equal to 0.49). Based on these input parameters, and using 1000 simulations, we calculated that a difference in change of NT-proBNP level over time of 6 ng/L per month can be demonstrated between cases and non-cases (α-error 0.05, power of 80%). This difference is small in clinical terms, demonstrating that the study has high statistical power.

Table 1 – Baseline characteristics

	Total (n=263)
	No. (%) / Mean (±SD) / Median (25th – 75th percentile)
Demographics	
Age	67 (±13)
Male gender	189 (72)
Caucasian ethnicity	244 (94)
Clinical characteristics	
Body mass index kg/m^2	28 (±5)
Heart rate, bpm	67 (±12)
Systolic blood pressure, mmHg	122 (±20)
Diastolic blood pressure, mmHg	73 (±11)
Biomarker level	
NT-proBNP (pmol/L)	137.3 (51.9 – 272.6)
HsTNT (ng/L)	18.0 (9.6 – 33.2)
CRP (mg/L)	2.2 (0.9 – 4.8)
Features of heart failure	
Duration of heart failure, years	4.6 (1.7 – 9.9)
NYHA class I or II	190 (73)
NYHA class III or IV	69 (27)
Left ventricular function	
Systolic dysfunction	250 (95)
HFPEF	13 (5)
LVEF*	32 (±10)
Etiology of heart failure	
Ischemic heart disease	117 (44)
Hypertension	34 (13)
Secondary to valvular heart disease	12 (5)
Cardiomyopathy	68 (26)
Dilated	49 (19)
Hypertrophic	12 (5)
Non compaction	4 (1)
Unclassified	3 (1)
Unknown	19 (7)
Other	13 (5)
Medical history	
Myocardial infarction	94 (36)
PCI	82 (31)
CABG	43 (16)
Valvular heart disease	136 (53)

Table 1 – Baseline characteristics (*continued*)

	Total (n=263)
	No. (%) / Mean (±SD) / Median (25th – 75th percentile)
Atrial fibrillation	105 (40)
Other arrhythmia	82 (32)
ICD	151 (59)
CRT	78 (30)
Pacemaker	38 (15)
CVA	41 (16)
Chronic renal failure	136 (53)
Diabetes Mellitus	81 (31)
Known hypercholesterolemia	93 (35)
Hypertension	120 (46)
Sleep apnea	26 (10)
Intoxications	
Alcohol consumption (>1 unit/day)	108 (42)
Smoking	185 (71)
Ever	186 (72)
Current	26 (10)
Medication use	
ACE-i	173 (67)
ARB	75 (29)
Aldosteron antagonist	178 (68)
Diuretic	237 (90)
Beta-blocker	232(88)
Aspirin	45 (17)
Vitamin K antagonist	200 (77)
Nitrates	44 (17)
Digoxin	59 (23)
Antiarrhythmics	39 (15)

Normally distributed continuous variables are presented as mean (± standard deviation). Non-normally distributed continuous variables are expressed as median (25^{th} – 75^{th} percentile). Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts, because of missing values. ACE-I = ace inhibitor; ARB = angiotensin II receptor blocker; CABG = coronary artery bypass grafting; COPD = chronic obstructive pulmonary disease; CRP = C-reactive protein; CRT = cardiac resynchronization therapy; CVA = cerebrovascular accident; HFPEF = heart failure with preserved ejection fraction; HsTNT = high sensitive cardiac troponin T; ICD = implantable cardioverter / defibrillator; LVEF = left ventricular ejection fraction; NT-proBNP = N-terminal pro-B-type natriuretic peptide NYHA = New York heart association; PCI = percutaneous coronary intervention; SD = standard deviation.

* Baseline echocardiograms were available in 72% of all patients because of logistic reasons.

RESULTS

Baseline findings

From October 2011 to August 2015, 263 patients were included. Baseline characteristics are shown in Table 1. Mean age of the study population was 67 years (SD ±12). The majority were men (72%) in New York heart association (NYHA) class I or II (73%). Median duration of HF was 4.6 years (IQR 1.7 – 9.9). Median baseline NT-proBNP was 137.3 pmol/L (IQR 51.9 – 272.6), HsTNT 18.0 ng/L (IQR 9.6 – 33.2) and CRP 2.2 mg/L (IQR 0.9 – 4.8). Positive associations were found between baseline NT-proBNP level and age (p=0.01), heart rate (p=0.01), NYHA class (p<0.001), and renal failure (p<0.001). Inverse associations were found between NT-proBNP and diastolic blood pressure (p<0.001) and BMI (p<0.001). Baseline HsTNT level was positively associated with age (p<0.001), NYHA class (p<0.001) and renal failure (p<0.001). Baseline CRP level showed positive associations with heart rate (p=0.01) and renal failure (p=0.045), and inverse associations with systolic (p=0.046) and diastolic blood pressure (p<0.001).

Clinical endpoints

During a median follow-up of 2.2 (IQR 1.4–2.5) years, 27 (10%) patients died from a cardiovascular cause, 56 (21%) patients were re-hospitalized for worsened HF, 5 (1.9%) patients underwent heart transplantation and 3 (1.1%) patients received LVAD-implantation (Table 2). Since 21 patients were re-hospitalized for worsened HF before dying from cardiovascular causes eventually during further follow-up, 70 patients (27%) reached the composite primary endpoint. Overall all-cause mortality was 32 (12%).

Associations between baseline characteristics and the primary endpoint are shown in Table 3. After multivariable adjustment, baseline NT-proBNP (HR 1.02; CI 1.01 – 1.02), baseline HsTNT (HR 1.08; CI 1.02 – 1.16), NYHA class (HR 1.61; CI 1.14 – 2.26) and diabetes mellitus type 2 (DM) (HR 1.91; CI 1.17 – 3.11) were independently associated with the primary endpoint (Table 3).

Temporal biomarker patterns and the primary endpoint

During follow-up, we collected 2193 blood samples, of which 2022 were drawn before the occurrence of the primary endpoint (median of 9 (IQR 5-10) samples per patient). The associations between the temporal biomarker patterns and the primary endpoint are shown in Table 4.

Table 2 – Endpoints

Endpoint	N (%)
Primary	
Combined primary endpoint*	70 (27)
Secondary	
Hospitalization for acute or worsening heart failure	56 (21)
All-cause mortality	32 (12)
Cardiovascular mortality	27 (10)
Heart transplantation	5 (1.9)
Left ventricular assist device implantation	3 (1.1)

Variables are displayed as count (percentage).

* The primary endpoint comprised heart failure hospitalization, cardiovascular mortality, cardiac transplantation and LVAD-implantation

Tabel 3 – Associations between baseline characteristics and the primary endpoint

Variabele	Crude HR (CI)	P	Adjusted HR (CI)‡	P
NT-proBNP (pmol/L)*	1.02 (1.02 – 1.03)	<0.001	1.02 (1.01 – 1.02)	<0.001
HsTNT (pg/mL)*	1.12 (1.08 – 1.16)	<0.001	1.08 (1.02 – 1.16)	0.020
CRP (mg/L)*	1.26 (1.06 – 1.50)	0.016	1.18 (0.96 – 1.45)	0.12
Age†	1.02 (1.01 – 1.05)	0.035	1.00 (0.98 – 1.02)	0.86
Male gender	1.27 (0.80 – 2.19)	0.40		
Systolic blood pressure†	0.99 (0.98 – 0.99)	0.040	0.99 (0.98 – 1.01)	0.26
Diastolic blood pressure†	0.98 (0.96 – 1.00)	0.055		
Heart rate†	1.01 (0.99 – 1.03)	0.24		
Body mass index kg/m^2†	1.00 (0.96 – 1.05)	0.88		
NYHA-class†	2.10 (1.56 – 2.54)	<0.001	1.61 (1.14 – 2.26)	0.006
Chronic renal failure	2.11 (1.28 – 3.50)	0.004	1.25 (0.72 – 2.18)	0.42
Diabetes mellitus	2.06 (1.29 – 3.29)	0.003	1.91 (1.17 – 3.11)	0.010
Hypercholesterolemia	1.37 (0.85 – 2.20)	0.20		
Hypertension	1.31 (0.82 – 2.10)	0.26		
Ever smoker	1.48 (0.84 – 2.62)	0.18		
History of CAD	1.56 (0.96 – 2.53)	0.074		
History of CVA	1.40 (0.78 – 2.51)	0.26		
ICD	1.20 (0.74 – 1.95)	0.47		
CRT	0.80 (0.47 – 1.36)	0.42		

CRP = C-reactive protein; CRT = cardiac resynchronization therapy; CVA = cerebrovascular accident; HsTNT = high sensitive cardiac troponin T; ICD = implantable cardioverter / defibrillator; NT-proBNP = N-terminal pro-B-type natriuretic peptide NYHA = New York heart association.

* HR per 10 units increase

† HR per unit increase

‡ All characteristics univariably associated with the primary endpoint ($p<0.05$) were entered into the multivariable Cox regression model.

Table 4 - Association between temporal patterns of logarithmically transformed NT-proBNP, hsTNT and CRP and the primary endpoint

	NT-proBNP		HsTNT		CRP	
	HR* (95% CI)	p-value	HR* (95% CI)	p-value	HR* (95% CI)	p-value
Temporal pattern of biomarker level						
Adjusted for age and gender	2.20 (1.83–2.65)	<0.001	2.21 (1.79–2.72)	<0.001	1.80 (1.43–2.26)	<0.001
Multivariable adjusted†	2.28 (1.82–2.86)	<0.001	2.05 (1.63–2.58)	<0.001	1.65 (1.30–2.08)	<0.001
Instantaneous slope of temporal pattern						
Adjusted for age and gender	2.16 (1.79–2.62)	<0.001	2.14 (1.71–2.66)	<0.001	1.85 (1.43–2.41)	<0.001
Multivariable adjusted†	2.15 (1.71–2.68)	<0.001	2.02 (1.58–2.58)	<0.001	1.69 (1.32–2.18)	<0.001
Area under the curve of temporal pattern						
Adjusted for age and gender	1.68 (1.45–1.96)	<0.001	1.74 (1.46–2.07)	<0.001	1.32 (1.13–1.55)	<0.001
Multivariable adjusted†	1.54 (1.30–1.83)	<0.001	1.55 (1.29–1.86)	<0.001	1.28 (1.09–1.51)	0.003
Three biomarkers combined						
Level, multivariable adjusted†	2.06 (1.53–2.79)	<0.001	1.41 (0.93–2.13)	0.104	1.38 (1.01–1.89)	0.047
Slope, multivariable adjusted†	2.04 (1.51–2.78)	<0.001	1.47 (0.94–2.16)	0.093	1.41 (1.02–1.94)	0.036
Area, multivariable adjusted†	1.99 (1.49–2.66)	<0.001	1.42 (0.94–2.13)	0.092	1.32 (0.96–1.80)	0.084

* HR = hazard ratio. Hazard ratios are given per doubling of level, slope or area under the curve at any point in time.

† adjusted for age, gender, BMI, renal function, NYHA class and diabetes mellitus type 2.

The temporal NT-proBNP pattern derived from the repeated measurements was a significant predictor of the primary endpoint after adjustment for age, gender, BMI, renal function, NYHA class and DM (HR per doubling of NT-proBNP: 2.28; CI 1.82 – 2.86; p<0.001). Figure 2a displays the curves depicting the temporal NT-proBNP pattern of patients who reached the primary endpoint versus those who did not.

Figure 2b depicts temporal HsTNT patterns of patients who reached the primary endpoint and those who did not. We found an association between the temporal HsTNT pattern and the primary endpoint, which remained present after multivariable adjustment (HR per doubling of biomarker: 2.05; CI 1.63 – 2.58; p<0.001).

As shown in figure 2c, the temporal CRP pattern was also a significant predictor of the primary endpoint (HR per doubling of CRP after multivariable adjustment: 1.65; CI 1.30 – 2.08; p<0.001).

NT-proBNP, HsTNT and CRP patterns and the primary endpoint using a combined 3-biomarker model

When we combined temporal patterns of all 3 biomarkers in 1 model, we found independent associations of NT-proBNP (HR per doubling of NT-proBNP level at any given time point: 2.06, CI 1.53-2.79, P b .001) and CRP (HR per doubling of CRP level: 1.38, CI 1.01-1.89, P = .047) with the primary end point. These associations were also independent of temporally changing total daily doses of equivalents of carvedilol, enalapril, furosemide, and spironolactone (Table IV). However, HsTNT was no longer associated with the primary end point in this model (HR per doubling of HsTNT: 1.41, CI 0.93-2.13, P =.10), illustrating that its predictive value was not independent of NT-proBNP and CRP.

Slopes and areas under the curve of temporal patterns

Table 4 displays hazard ratios for the doubling of the instantaneous slopes and areas under the curve of the temporal biomarker patterns. The instantaneous slopes of the temporal patterns as well as the areas under the curve of NT-proBNP, HsTNT and CRP were all associated with the primary endpoint after multivariable adjustment.

When we entered the instantaneous slopes of the temporal biomarker patterns of the 3 biomarkers into 1 model, they remained independent predictors for NT-proBNP and CRP, but not for HsTNT. Simultaneously entering the areas under the curve of the 3 temporal biomarker patterns into 1 model showed that only NT-proBNP was independently associated with the primary endpoint.

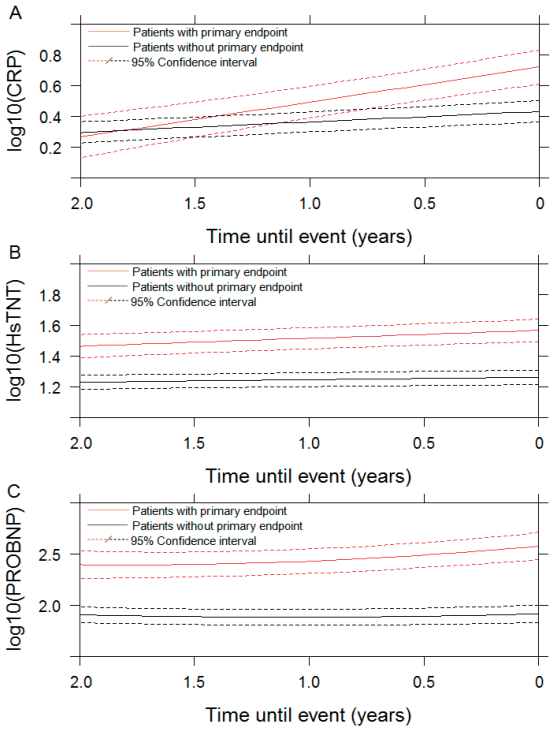


Figure 2 - Temporal patterns

The temporal patterns, displayed as time until event, of logarithmically transformed A) N-terminal pro B-type natriuretic peptide (NT-proBNP), B) high-sensitive troponin T (HsTNT) and C) C-reactive protein (CRP) of patients who reached the primary endpoint versus those who did not.

Personalized prediction: individual, dynamic risk estimation

Figure 3 shows the temporal patterns of the biomarkers in several individual patients from our data set together with their corresponding individual probabilities of survival as estimated by the joint model. The figure shows that each time an additional measurement is performed in the patient, the individual probability of survival is updated. Specifically, rising marker levels and worsening prognosis can be seen in the example patients who ultimately reached the composite end point versus stable or decreasing marker levels and more favourable prognosis in the example patients who stayed event-free. These individual estimates of prognosis can be obtained by clinicians in an easy, user-friendly manner. Joint models, like those we have constructed, can be uploaded into an app (http://shiny.rstudio.com/) that creates an interface into which a clinician can add the characteristics, and consecutive biomarker measurements, of an individual patient. Subsequently, the app returns the curve depicting individual prognosis (Supplemental Figure 1).

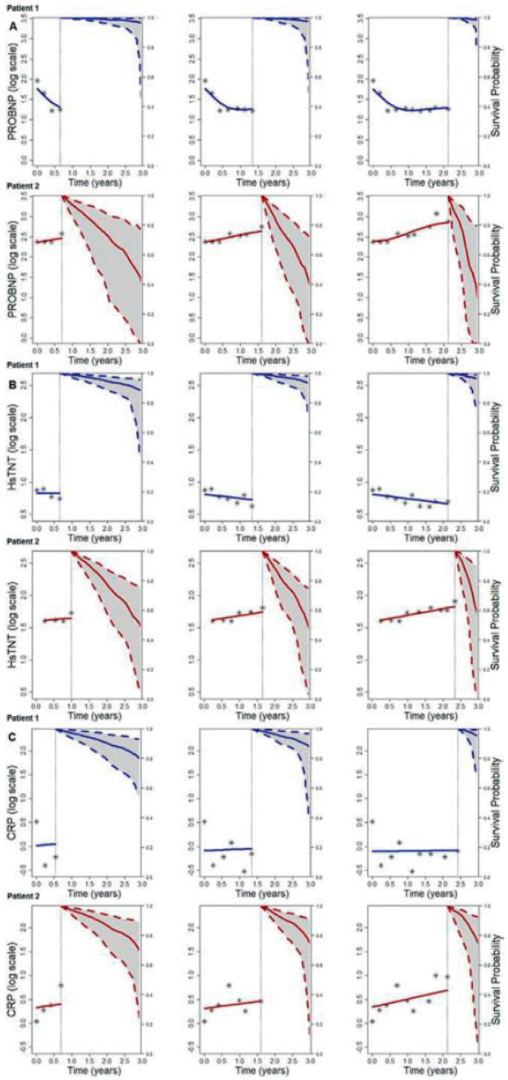


Figure 3. Dynamic profiling of an individual patient's risk using patient-specific temporal trajectories.

The solid red lines depict patients who experienced the study endpoints, and the solid blue lines depict patients who did not.

The X-axis depicts follow-up time starting from baseline. Biomarker levels (on the log scale) are displayed on the left Y-axis and survival probability (%) on the right Y-axis. Patient-specific temporal biomarker trajectories are displayed left of the vertical dotted black line. To the right of this line, the corresponding conditional survival probability curve is displayed with 95% confidence intervals (grey area). To show this conditional survival probability curve is dynamically updated every time an extra measurement is recorded, we provide the curves for three time-points at which risk was updated.

Model performance

Discriminative ability of models containing the temporal patterns of the biomarker levels and baseline measurements only is shown in table 5. For all 3 biomarkers, models containing temporal biomarker patterns showed higher c-indices than those containing baseline measurements only. The highest c-index resulted from the multivariable model containing all 3 temporal biomarker patterns as well as age, gender, BMI, renal function, NYHA class and DM (c-index 0.84).

Table 5 – Discriminative ability of models containing the temporal patterns of NT-proBNP, HsTNT and CRP level, as well as models containing baseline measurements only

	Baseline measurements	Temporal patterns
	c-index; multivariable model*	c-index; multivariable model*
NT-proBNP	0.78	0.83
HsTNT	0.73	0.75
CRP	0.67	0.69
Combined model†	0.79	0.84

* The multivariable models were corrected for: age, gender, BMI, renal function, NYHA class and diabetes mellitus type 2.

† NT-proBNP, HsTNT and CRP are all included in the combined model.

Temporal patterns of NT-proBNP, HsTNT and CRP in relation to hospitalisation for acute or worsening HF (secondary endpoint)

All three biomarker patterns were strong individual predictors of HF hospitalisations (age- and gender adjusted HRs per doubling of biomarker: NT-proBNP, 2.17; CI 1.57 – 2.66; p<0.001; HsTNT, 2.18; CI 1.72 – 2.76; p<0.001 and CRP, 1.99; CI 1.53 – 2.59; p<0.001). These associations remained statistically significant after multivariable adjustment (HRs per doubling of biomarker: NT-proBNP, 2.31; CI 1.77 – 3.01; p<0.001; HsTNT, 1.95; CI 1.49 – 2.55; p<0.001 and CRP, 1.80; CI 1.37 – 2.35; p<0.001). After creating a time-dependent Cox model using all 3 temporal biomarkers patterns, derived from the individual joint models, we found that each of the 3 biomarkers remained independent predictors of HF hospitalizations (HR per doubling of biomarker: NT-proBNP, 1.51; CI 1.26 – 1.80; p<0.001; HsTNT, 1.57; CI 1.24 – 2.00; p=0.001 and CRP, 1.41; CI 1.15 – 1.74; p<0.001). These associations persisted after adjusting for temporally changing total daily doses of equivalents of carvedilol, enalapril, furosemide, and spironolactone (HR per doubling of biomarker: NT-proBNP 1.49, CI 1.23-1.80, P b .001; HsTNT 1.50, CI 1.18-1.91, P = .001; and CRP 1.39, CI 1.11-1.74, P = .004).

DISCUSSION

We performed a prospective, observational study that comprised CHF patients with mostly systolic dysfunction and predominantly favourable NYHA class (I-II). Here, in the first inclusion round, we demonstrate that the dynamic, temporal patterns of serially measured NT-proBNP and CRP levels are strong and independent predictors of adverse clinical events. Moreover, instantaneous slope of these biomarkers' temporal trajectories, as well as the area under the curve of their temporal trajectories, is associated with adverse events. The temporal patterns of HsTNT also significantly predict adverse events but lose their predictive capability when combined with temporal NT-proBNP and CRP patterns.

We also demonstrate, based on these dynamic models, how individual, temporal biomarker trajectories can be used for calculating patient-specific risk estimates, which are dynamically updated every time a patient has a new measurement performed.

Studies on the prognostic value of repeated natriuretic peptide measurements have mostly been performed in trial participants,6,13 and studies on the prognostic value of repeated biomarker measurements other than natriuretic peptides are scarce.3,4,12 Altogether, these existing studies describing temporal changes in biomarkers in relation to patient prognosis have 3 major limitations. Firstly, changes are often presented as a difference between just 2 measurements that are separated in time. Such an approach fails to fully capture the true biomarker pattern of the dynamic disease. Moreover, it fails to expose changes in biomarker level prior to clinically relevant end points because, on average, a long time period lies between the last (i.e., second) biomarker measurement and the incident end point. To properly investigate whether an increase in biomarker level is present at the time an end point is approaching and whether this increase truly contributes to an individual's risk, the time period between the last measurement and the end point should be kept as brief as possible. This implies that a high frequency of blood sampling during prolonged follow-up is needed. Secondly, biomarkers are often studied in isolation, thus actually ignoring the different underlying etiologies that converge to adverse cardiac remodeling and HF progression. The third limitation of existing studies is related to the applied methods of data analysis. Often, absolute or relative differences between 2 measurements are calculated, or categorical changes across a threshold value are assessed. These various approaches to temporal change all render different estimates for associations between changes in biomarker level and outcome,1 which are an illustration of their shortcomings. At best, Cox models with

so-called time-dependent covariates are used to analyze the effects of temporal biomarker patterns. Although time- dependent Cox models assume that biomarker levels do not change between measurements, it is known that biomarker patterns are dynamic and continuously changeover time, parallel to the condition of the patient. All these limitations are overcome in Bio-SHiFT: we have performed a large number of frequent, repeated measurements (up to 11 trimonthly samples per patient); we have studied multiple biomarkers; and we have applied modern statistical methods ("joint modeling"), which, as stated above, take into account the continuous, dynamic changes in biomarker patterns and thus result in less bias. 21

Several randomized trials have been performed to investigate whether using serial natriuretic peptide measurements to titrate medical therapy can improve clinical outcome of HF patients. However, because the results of these trials were not fully consistent, natriuretic peptide–guided therapy remains controversial.8-10,15

It should be noted that most of these trials were based on protocols that used uniform natriuretic peptide targets in the intervention groups.22-26 Existing trials that used individualized treatment targets are in the minority and often based their targets on natriuretic peptide levels that were measured briefly after the index episode of decompensation when titration of therapy was still ongoing.27-29 Conversely, our study describes in detail the temporal biomarker patterns in stable CHF patients and reveals significant associations between temporal patterns of biomarker levels and adverse events. Patients with CHF who did not experience adverse cardiac events during prolonged follow-up were shown to have lower levels of NT-proBNP, HsTNT, and CRP at any moment in time compared with patients who did experience adverse cardiac events during follow-up. Additionally, the instantaneous rate of change in biomarker levels (represented by the slope of the temporal biomarker patterns), as well as the cumulative values the marker has taken in the past (represented by the area under the temporal biomarker patterns), was associated with adverse outcome. These findings support the concept of an individualized biomarker target level instead of a generally applicable uniform cut off value for all patients. On top of this, they suggest that rate of change in biomarker level and the duration of biomarker level elevation merit attention to provide appropriate individual treatment targets as well as correct estimates of prognosis. Our study also demonstrates that temporal patterns of CRP predict adverse clinical outcome independently of NT-proBNP.

Future trials on biomarker-guided therapy may benefit from incorporating these findings. Firstly, future trials should use personalized biomarker cut off values, that

is, interpret a patient's biomarkers level in the context of his or her previous series of levels. This means that they should not only take into account the absolute biomarker level but also incorporate the instantaneous slope of the marker's trajectory. Secondly, upcoming trials should use a combination of multiple biomarkers, representing different pathophysiological pathways, to guide HF therapy. Finally, additional research should be performed on the frequency of biomarker measurement and tailoring thereof to individual patients; subsequently, these findings should be incorporated into biomarker-guided trials as well.

Miller et al 12 published a study that might be considered comparable to Bio-SHiFT to a certain extent, as they evaluated serial measurements of cardiac troponin T and BNP in 190 ambulant CHF patients. Again, an important limitation of this study is the use of time-dependent Cox models. Still, Miller et al found that cardiac troponin T and BNP were both independent predictors of cardiac mortality or cardiac transplantation and that combined elevation of these biomarkers substantially adds to risk. We could only partly confirm these results. In Bio-SHiFT, although predictive as a separate marker, the HsTNT pattern appeared to be no longer significantly associated with the primary study end point after adjustment for the NT-proBNP and CRP patterns (and also after adjustment for NT-proBNP alone; data not shown). This may (at least in part) be due to the above-described differences in data analysis.

Some aspects of this study warrant consideration. With 263 patients, sample size is limited, and the majority of the patient population was in NYHA class I or II and had systolic dysfunction. Also, a large proportion had concomitant valvular heart disease. The results and conclusions should be judged accordingly because such a study population may not be fully representative of "real-life" CHF patients in general. Nevertheless, given the repeated-measures design, N2,000 blood samples were available, and all 3 investigated biomarkers, each having different pathophysiological properties, showed the hypothesized rising temporal pattern. This strengthens our findings and makes them less likely attributable to bias or chance. Further to this, the current investigation was an interim analysis of the patients enrolled in the first inclusion round. The full Bio-SHiFT cohort was designed to enroll 400 patients and to have sufficient statistical power to perform large-scale, hypothesis-free research on novel, lesser known biomarkers. In such cases, correction for multiple testing is warranted. The current investigation, however, examines 3 well-established biomarkers, which have been extensively implicated in HF in previous studies and which were chosen based on pathophysiological considerations, rendering correction for multiple testing redundant. Furthermore, additional investigations are needed to

estimate the most efficient frequency of biomarker measurement so that optimal prognostic information can be gained without superfluous blood sampling. In our study, patients were monitored every 3 months to construct a data framework for our joint models. An extension to joint modeling is currently being developed to define optimal time frames for individual patients to return for consecutive measurements. In this context, the optimal frequency for biomarker measurement is expected to vary from patient to patient; it is likely that once a stable biomarker value is found in a patient, this patient could be re-examined after a longer time period, whereas if, for example, a biomarker value is found to have risen and thus prognosis is worsening, the patients may need to return more quickly. Moreover, in our study, repeatedly measured NT-proBNP and CRP were both independently associated with the primary end point. This implies that a multi- marker model would benefit monitoring of CHF patients (although a model combining both of these biomarkers seemed to have little incremental discriminative value over serial NT-proBNP assessment only as suggested by the C-index, the C-index is known to be rather insensitive to improvements in prediction performance, and it has been demonstrated previously that testing for improvement in prediction performance is actually redundant if a variable has already been shown to be an independent risk factor 30). Future studies should investigate a broader spectrum of biomarkers to further improve risk assessment. Finally, although we have illustrated the application of biomarker-guided, personalized risk assessment in practice by means of an interface that uses joint modeling, we realize that many challenges remain to be resolved before truly implementing such a strategy.

In conclusion, detailed temporal patterns of NT-proBNP and CRP are strong, independent predictors of adverse clinical events in our study population of patients with stable CHF. Not only evolution of biomarker level but also instantaneous rate of change in level of NT-proBNP and CRP as well as the area under the curve of the trajectory of NT-proBNP was associated with adverse outcome. These findings suggest that individual patterns of change of biomarkers, as well as combinations of multiple biomarkers, should be taken into consideration for prognostication in patients with stable CHF. Overall, our study illustrates that several aspects of biomarker- guided risk stratification have been incompletely ad- dressed so far and that there still seems to be room for improvement with regard to personalized risk assessment. Future steps could potentially include determining optimum timing of blood sampling, determining optimum combinations of biomarkers, and eventu-

ally a biomarker-guided trial that is based on personalized temporal patterns of multiple biomarkers.

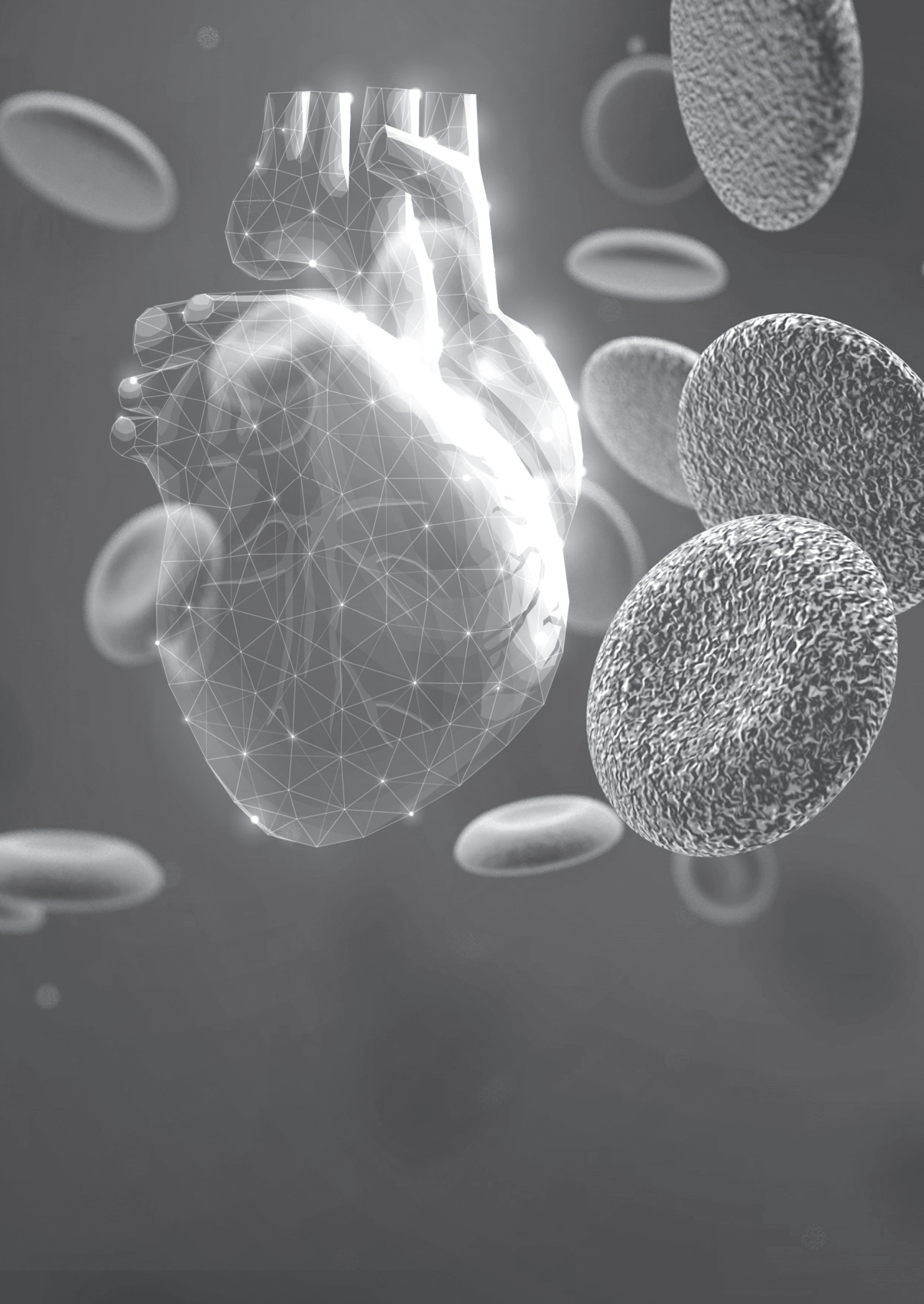
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ahj.2017.10.008.

REFERENCES

1. Masson S, Latini R, Anand IS, et al. Prognostic value of changes in N-terminal pro-brain natriuretic peptide in Val-HeFT (Valsartan Heart Failure Trial). J Am Coll Cardiol 2008;52:997-1003.
2. Anand IS, Latini R, Florea VG, et al. C-reactive protein in heart failure: prognostic value and the effect of valsartan. Circulation 2005;112:1428-1434.
3. Sato Y, Yamada T, Taniguchi R, et al. Persistently increased serum concentrations of cardiac troponin t in patients with idiopathic dilated cardiomyopathy are predictive of adverse outcomes. Circulation 2001;103:369-374.
4. Perna ER, Macin SM, Canella JP, et al. Ongoing myocardial injury in stable severe heart failure: value of cardiac troponin T monitoring for high-risk patient identification. Circulation 2004;110:2376-2382.
5. Bettencourt P, Azevedo A, Pimenta J, et al. N-terminal-pro-brain natriuretic peptide predicts outcome after hospital discharge in heart failure patients. Circulation 2004;110:2168-2174.
6. Anand IS, Fisher LD, Chiang YT, et al. Changes in brain natriuretic peptide and norepinephrine over time and mortality and morbidity in the Valsartan Heart Failure Trial (Val-HeFT). Circulation 2003;107:1278-1283.
7. O'Brien RJ, Squire IB, Demme B, et al. Pre-discharge, but not admission, levels of NT-proBNP predict adverse prognosis following acute LVF. Eur J Heart Fail. 2003;5:499-506.
8. Felker GM, Hasselblad V, Hernandez AF, et al. Biomarker-guided therapy in chronic heart failure: a meta-analysis of randomized controlled trials. Am Heart J 2009;158:422-430.
9. Savarese G, Trimarco B, Dellegrottaglie S, et al. Natriuretic peptide-guided therapy in chronic heart failure: a meta-analysis of 2,686 patients in 12 randomized trials. PLoS One. 2013;8:e58287.
10. Porapakkham P, Porapakkham P, Zimmet H, et al. B-type natriuretic peptide-guided heart failure therapy: A meta-analysis. Arch Intern Med 2010;170:507-514.
11. Latini R, Masson S, Anand IS, et al. Prognostic value of very low plasma concentrations of troponin T in patients with stable chronic heart failure. Circulation 2007;116:1242-1249.
12. Miller WL, Hartman KA, Burritt MF, et al. Serial biomarker measurements in ambulatory patients with chronic heart failure: the importance of change over time. Circulation 2007;116:249-257.
13. Yan RT, White M, Yan AT, et al. Usefulness of temporal changes in neurohormones as markers of ventricular remodeling and prognosis in patients with left ventricular systolic dysfunction and heart failure receiving either candesartan or enalapril or both. Am J Cardiol 2005;96:698-704.
14. Maisel A, Barnard D, Jaski B, et al. Primary results of the HABIT Trial (heart failure assessment with BNP in the home). J Am Coll Cardiol 2013;61:1726-1735.
15. Troughton RW, Frampton CM, Brunner-La Rocca HP, et al. Effect of B-type natriuretic peptide-guided treatment of chronic heart failure on total mortality and hospitalization: an individual patient meta-analysis. Eur Heart J 2014;35:1559-1567.

16. Adams KF, Jr., Felker GM, Fraij G, et al. Biomarker guided therapy for heart failure: focus on natriuretic peptides. Heart Fail.Rev 2010;15:351-370.
17. Dickstein K, Cohen-Solal A, Filippatos G, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur Heart J 2008;29:2388-2442.
18. McMurray JJ, Adamopoulos S, Anker SD, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 2012;33:1787-1847.
19. Paulus WJ, Tschope C, Sanderson JE, et al. How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. Eur Heart J 2007;28:2539-2550.
20. Rizopoulos D. JM: An R Package for the Joint Modelling of Longitudinal and Time-to-Event Data. Journal of Statistical Software 2010;35:1-33.
21. Rizopoulos D, Takkenberg JJ. Tools & techniques--statistics: Dealing with time-varying covariates in survival analysis--joint models versus Cox models. Eurointervention 2014;10:285-288.
22. Troughton RW, Frampton CM, Yandle TG, et al. Treatment of heart failure guided by plasma aminoterminal brain natriuretic peptide (N-BNP) concentrations. Lancet 2000;355:1126-1130.
23. Jourdain P, Jondeau G, Funck F, et al. Plasma brain natriuretic peptide-guided therapy to improve outcome in heart failure: the STARS-BNP Multicenter Study. J Am Coll Cardiol 2007;49:1733-1739.
24. Pfisterer M, Buser P, Rickli H, et al. BNP-guided vs symptom-guided heart failure therapy: the Trial of Intensified vs Standard Medical Therapy in Elderly Patients With Congestive Heart Failure (TIME-CHF) randomized trial. JAMA 2009;301:383-392.
25. Januzzi JL, Jr., Rehman SU, Mohammed AA, et al. Use of amino-terminal pro-B-type natriuretic peptide to guide outpatient therapy of patients with chronic left ventricular systolic dysfunction. J Am Coll Cardiol 2011;58:1881-1889.
26. Berger R, Moertl D, Peter S, et al. N-terminal pro-B-type natriuretic peptide-guided, intensive patient management in addition to multidisciplinary care in chronic heart failure a 3-arm, prospective, randomized pilot study. J Am Coll Cardiol 2010;55:645-653.
27. Shah MR, Califf RM, Nohria A, et al. The STARBRITE trial: a randomized, pilot study of B-type natriuretic peptide-guided therapy in patients with advanced heart failure. J Card Fail. 2011;17:613-621.
28. Eurlings LW, van Pol PE, Kok WE, et al. Management of chronic heart failure guided by individual N-terminal pro-B-type natriuretic peptide targets: results of the PRIMA (Can PRo-brain-natriuretic peptide guided therapy of chronic heart failure IMprove heart fAilure morbidity and mortality?) study. J Am Coll Cardiol 2010;56:2090-2100.

29. Persson H, Erntell H, Eriksson B, et al. Improved pharmacological therapy of chronic heart failure in primary care: a randomized Study of NT-proBNP Guided Management of Heart Failure--SIGNAL-HF (Swedish Intervention study--Guidelines and NT-proBNP AnaLysis in Heart Failure). Eur J Heart Fail. 2010;12:1300-1308.



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Serially measured circulating miR-22-3p is a biomarker for adverse clinical outcome in patients with chronic heart failure: The Bio-SHiFT study

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Serially measured circulating miR-22-3p is a biomarker for adverse clinical outcome in patients with chronic heart failure: The Bio-SHiFT study

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ABSTRACT

Background: Several studies have suggested circulating microRNAs (miRs) are associated with heart failure, but these studies were small, and limited to single miR measurements. We examined 7 miRs which were previously linked to heart failure, and tested whether their temporal expression level predicts prognosis in a prospective cohort of chronic heart failure (CHF) patients.

Methods: In 2011-2013, 263 CHF patients were included. At inclusion and subsequently every 3 months, we measured 7 miRs. The primary endpoint (PE) comprised heart failure hospitalization, cardiovascular mortality, cardiac transplantation and LVAD implantation. Associations between temporal miR patterns and the PE were investigated by joint modelling, which combines mixed models with Cox regression.

Results: Mean age was 67±13 years, 72% were men and 27% NYHA class III–IV. We obtained 873 blood samples (median 3 [IQR 2-5] per patient). The PE was reached in 41 patients (16%) during a median follow-up of 0.9 [0.6-1.4] years. The temporal pattern of miR-22-3p was independently associated with the PE (HR [95% CI] per doubling of level: 0.64 [0.47 - 0.77]). The instantaneous change in level (slope of the temporal miR pattern) of miR-22-3p was also independently associated with the PE (HR [95% CI] per doubling of slope: 0.33 [0.20–0.51]). These associations remained statistically significant after adjustment for temporal patterns of NT-proBNP, Troponin T and CRP.

Conclusions: The temporal pattern of circulating miR-22-3p contains important prognostic and independent information in CHF patients. This concept warrants further investigation in larger series with extended follow-up.

INTRODUCTION

Contemporary treatment of chronic heart failure ((C)HF) generally aims to stabilize or at least decelerate disease progression. Adjustment of pharmacotherapy is largely based on clinical judgement and thus mostly relies on the worsening of symptoms or signs.1,2 However, in the context of the complexity of HF therapy, considerable clinical skills, as well as cooperation of the patients, are required to recognize opportunities to titrate therapies and to implement such changes intervene early and timely.3 Consequently, higher-risk patients may be undertreated.3 Blood biomarkers have the potential to monitor subtle changes in the heart that reflect and possibly predict adverse changes before they become clinically apparent or reported.4 The value of biomarkers, such as B-type natriuretic peptides (BNP), cardiac troponins and C- reactive protein (CRP), for risk stratification of CHF patients has already been demonstrated.5-8 Moreover, natriuretic peptide-guided HF therapy has recently been given a class IIa recommendation in US HF guidelines to achieve guideline-directed medical therapy.2,9

Nevertheless, the predictive capability of the aforementioned biomarkers for worsening of CHF still leaves room for improvement. MicroRNAs (miRs) are upcoming novel biomarkers that seem promising for early diagnosis and treatment of HF. MiRs are non-coding, ~22 nucleotide long RNA sequences, which target messenger RNAs for cleavage or translational repression and thereby influence a great variety of biological processes.10 The stability of miRs in plasma, and consequently their reliable assessment in easily accessible samples, potentially makes them attractive biomarkers for a wide range of diseases.11 Studies revealing that deletion of Dicer, a gene encoding an RNase III endonuclease essential for miR processing, leads to cardiac remodeling and dilation, were the first to show involvement of miRs in HF12,13 and to suggest that miRs might be used as biomarkers for cardiovascular diseases.14 Several other studies have subsequently shown associations between miRs and myocardial infarction15-18 and HF.19-24 However, most studies pertaining to HF were performed in case-control settings and had a limited sample size. Furthermore, these studies usually assessed miRs only once. Repeated, longitudinal miR assessment in CHF patients may, however, provide insight into individual, tem-

poral patterns and the patient's ensuing risk of disease progression. The temporal patterns of miRs in patients with known CHF have not yet been investigated.

In the current prospective, observational study, we have performed frequent (up to 8), repeated measurements of multiple miRs that were previously linked to HF (miR-1254, miR-22-3p, miR-423-5p, miR-486-5p and miR-320a) or have been shown to be cardiac-enriched (miR-345-5p, miR-378a-3p) in a cohort of 263 outpatients with CHF, and have subsequently investigated the associations of the obtained temporal patterns with adverse clinical outcome during follow-up.

METHODS

Patients

The Serial biomarker measurements and new echocardiographic techniques in chronic heart failure patients result in tailored prediction of prognosis (Bio-SHiFT) study was designed to investigate the relationship between temporal patterns of biomarkers involved in CHF and prognosis. Bio-SHiFT is an ongoing prospective, observational study of stable outpatients with CHF, conducted in Erasmus MC, Rotterdam, The Netherlands and Medical Centre Alkmaar, The Netherlands. Patients were recruited during their regular outpatient visits and were in clinically stable condition. Patients were eligible for inclusion if aged 18 years or older, capable of understanding and signing informed consent, and if CHF (including HF with preserved ejection fraction (HFPEF)) was diagnosed ≥ 3 months ago according to the guidelines of the European Society of Cardiology (ESC).25-27 Detailed inclusion and exclusion criteria are shown in figure 1. The study was approved by the medical ethics committees of the participating hospitals and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. The study is registered in ClinicalTrials.gov, number NCT01851538. In the current paper, we have performed an analysis on the 263 patients who were enrolled during the first inclusion period between October 2011 and June 2013. Follow-up for this analysis lasted from October 2011 until November 2013.

Baseline assessment

At baseline patients were evaluated by trained research physicians, who collected information on HF related symptoms including NYHA class25,26 and performed a physical examination, including blood pressure, heart rate and body mass

index. Information on aetiology of heart failure, presence of systolic dysfunction, cardiovascular risk factors, medical history and medical treatment was retrieved primarily from hospital records and was completed by anamnesis in case of ambiguities. History of myocardial infarction, percutaneous coronary intervention (PCI), coronary artery bypass grafting, valvular heart disease, atrial fibrillation or other arrhythmias, device implantation, cerebrovascular accident, diabetes mellitus, hypercholesterolemia, hypertension, and sleep apnea were defined as a clinical diagnosis of these conditions, as reported by the treating physician in the medical chart. History of chronic renal failure was defined as glomerular filtration rate less than 60 mL/min/1.73 m2. Alcohol consumption was defined as drinking ≥1 alcoholic consumption per day. Electrocardiography and echocardiography were performed. Data were entered into electronic case report forms. Non-fasting blood and urine samples were collected.

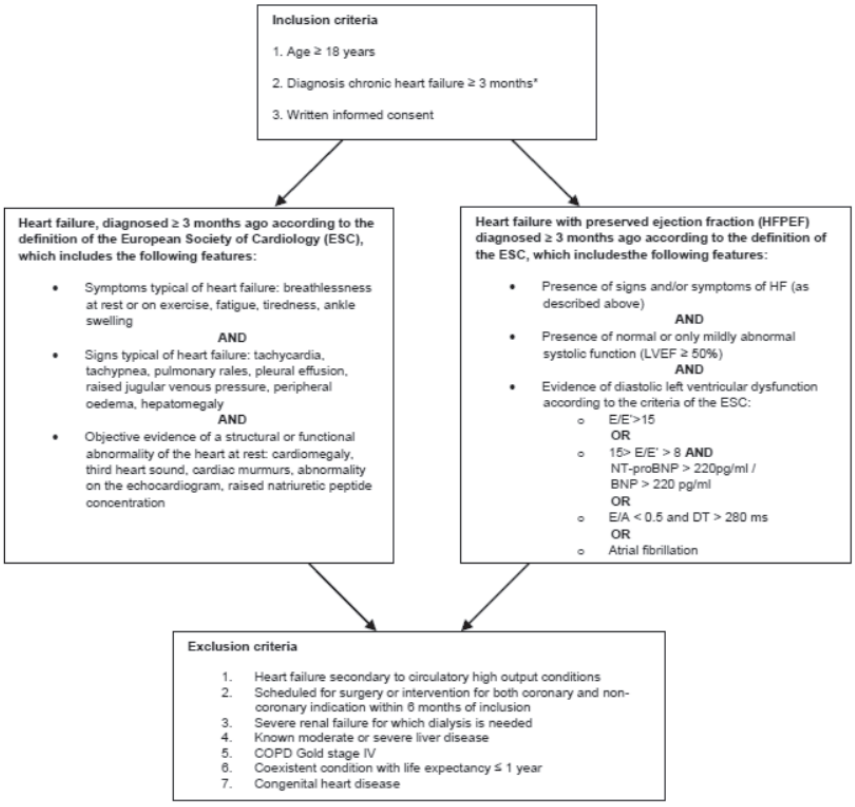


Figure 1 - Inclusion and exclusion criteria

Follow-up visits

All patients continued routine outpatient visits during the study. Study follow-up visits were scheduled every 3 months (a window of ±1 month was allowed), for a maximum of 30 months. For patients' convenience, study visits and routine outpatient visits were combined when possible. At each tri-monthly study visit, a short medical evaluation was performed and blood and urine samples were collected. Adverse cardiovascular events and changes in medication were recorded in electronic case report forms.

Blood sampling and miR measurement

Blood samples were collected at baseline and at each follow-up visit, and were processed and stored locally at a temperature of -80oC within 2 hours after blood collection. When applicable, samples were transported to the central laboratory (Erasmus MC, Rotterdam, The Netherlands) under controlled conditions (at a temperature of -80oC), until batch analysis took place. Accordingly, results of the biomarker assays were not available to treating physicians at the time of the outpatient visits. Thus, the biomarker measurements did not lead to treatment adjustments, and all patients received usual care based on European guidelines.25,26

For the purpose of the current investigation, stored plasma samples were transported under controlled conditions to ACS Biomarker, Amsterdam, The Netherlands, and seven miRs were measured in one batch: miR-1254, miR-22-3p, miR-345-5p, miR-378a-3p, miR-423-5p, miR-486-5p and miR320a. MiR-1254, miR-22-3p, miR-423-5p, miR-486-5p and miR-320a were selected because they were associated with HF in previous studies.11,21,24 MiR-378a-3p and miR-345-5p were selected because of their presence in cardiomyocytes.28 Plasma was thawed on ice and RNA isolation was performed using the TRIZOL LS reagent (Life Technology) according to the manufacturer's protocol, with a starting volume of 200ul plasma. Subsequently, 8ul of the eluate from the RNA isolation was used for the reverse transcription reaction. The transcription followed the manufacturer's protocol of the miScript Reverse Transcription Kit (Qiagen). For real-time PCR, 2ul of 10x diluted cDNA was used in a total volume of 10ul. The real-time PCR reaction was performed on a LightCycler 480 using the following program: 5 min of pre-incubation at 95°C; 10 sec of denaturation at 95°C, 20 sec of annealing at 58°C and 30 sec of elongation at 72°C, in a total of 45 cycles. Data were analyzed with LinRegPCR quantitative PCR data analysis software. MiR values were normalized using exogenous C. elegans miR-39 as a spike-in control, which was applied prior to the RNA isolation step. The

forward primers used were: miR-423-5p: TGAGGGGCAGAGAGCGAGACTTT; miR-22-3p: AAGCTGCCAGTTGAAGAACTGT; miR-378a-3p: ACTGGACTTGGAGTCAGAAGG; miR-1254: CTGGAAGCTGGAGCCTGC; miR-345-5p: GCTGACTCCTAGTCC; miR-486-5p: TCCTGTACTGAGCTG; miR-320a: AAAAGCTGGGTTGAGAGGGCGA.

Batch analysis of N-terminal pro B-type natriuretic peptide (NT-proBNP), high-sensitive cardiac troponin T (HsTNT) and C-reactive protein was performed in the Clinical Chemistry Laboratory of the Erasmus MC, Plasma NT–proBNP and cardiac Troponin T were analyzed using electrochemiluminescence immunoassays (Roche Diagnostics, Elecsys 2010, Indianapolis, Indiana, USA), measuring concentrations ranging from 5 to 35000 ng/L and 3 to 10000 ng/L, respectively. CRP was measured using an immunoturbidimetric assay (Roche Hitachi 912 chemistry analyzer, Basel, Switzerland), which measures concentrations ranging from 0.3 to 350 mg/L.

Clinical study endpoints

During follow-up, endpoints were recorded in the electronic case report forms by trained research physicians, and associated hospital records and discharge letters were collected. A clinical event committee blinded to the biomarker results subsequently reviewed all collected information and adjudicated primary and secondary endpoints.

The primary endpoint comprised the composite of cardiac death, cardiac transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened HF. Secondary endpoints included individual components of the primary endpoint, as well as myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting, cerebrovascular accident, and all-cause mortality.

Cardiac death was defined as death from myocardial infarction or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalization for acute or worsened HF was defined as exacerbation of symptoms typical of HF, in combination with 2 of the following: BNP or NT-proBNP >3x ULN, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.

Statistical analysis

Distributions of continuous variables, including biomarker and miR concentrations, were tested for normality using the Kolmogorov-Smirnov test. Variables with normal distributions are presented as mean±SD. Variables with non-normal distributions are presented as median and interquartile range (IQR). Categorical data are displayed as count and percentage.

In case of skewed distributions, continuous variables were logarithmically transformed for further analyses. Associations between temporal patterns of each separate miR and the primary endpoint were assessed using a joint modelling approach, which combines a linear mixed-effects model for the serial miR measurements with a Cox proportional hazards model for the occurrence of the primary endpoint. Results are presented as hazard ratios (HRs) per doubling of the miR concentration at any point in time, along with the corresponding 95% CIs. All analyses were adjusted for age and gender. Subsequently, additional multivariable adjustment for potential confounding variables was performed. For this purpose, associations between patient characteristics and baseline miR levels, as well as associations between patient characteristics and the primary endpoint, were identified using univariable linear- or Cox regression analyses, when appropriate. All characteristics associated with the miRs or the primary endpoint were subsequently used as covariates in the mixed-effects and Cox models used to construct the joint models. Adjustment for repeatedly assessed NYHA class was performed by entering NYHA class as a time-dependent variable into the joint model.

The above-described analysis assesses the predictive value of the temporal pattern of the actual miR measurements. However, additional features of the miR's trajectory may better predict the primary endpoint.29,30 Therefore, we investigated whether the instantaneous rate of change in miR (slope of the miR trajectory) is associated with the risk of the primary endpoint. Furthermore, to assess the predictive value of long-term elevation of miR levels, we investigated the associations between the area under the miRs' trajectory and the primary endpoint. This area can be considered as a weighted average of the total longitudinal profile of each patient. The results are presented as HRs per doubling of the area at any point in time, with 95% CIs.

Subsequently, we assessed whether the predictive value of the temporal miR patterns was independent of temporal patterns of NT-proBNP, HsTNT and CRP. For this purpose, all individual temporal miR patterns (of miRs significantly associated with the primary endpoint), as well as NT-proBNP, HsTNT and CRP patterns, derived from

the adjusted joint models were saved and subsequently entered simultaneously as time-varying covariates into an extended Cox analysis. The same approach was used to investigate the independent predictive value of the slope and the area under the curve of the temporal miR patterns associated with the primary endpoint.

To investigate the associations between repeated miR measurements and repeated NT-proBNP, HsTNT and CRP measurements, we used generalised estimating equations. Herewith, we examined whether the miR level at a certain time-point is associated with the level of the other biomarkers at that same time-point.

All analyses were performed with R Statistical Software using package JM.29,30 All tests were two-tailed and p-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics

From October 2011 to June 2013, 263 patients were included. Mean age was 67 years (SD ±13), 72% were men, and 73% were in New York Heart Association (NYHA) class I or II (Table 1). Median baseline NT-proBNP was 139.6 pmol/L (IQR 51.9 – 272.9), HsTNT 18.0 ng/L (IQR 9.6 – 33.2) and CRP 2.2 mg/L (IQR 0.9 – 4.8). Median duration of HF at inclusion was 4.6 years (IQR 1.7 – 9.9). Associations between patient characteristics and baseline miR measurements are presented in the supplementary table. Of note is the presence of a significant association between ischemic cardiomyopathy (ICM) and several miRs. This is further illustrated in figure 2. No significant associations were present with other baseline characteristics.

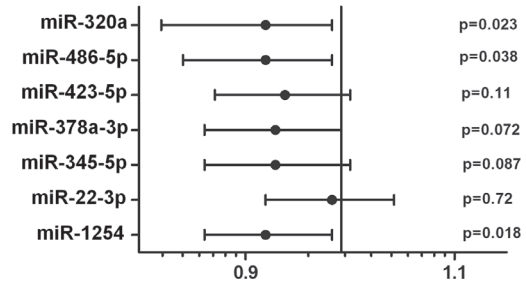


Figure 2 - Associations between baseline MicroRNA levels and presence of ischemic cardiomyopathy
Odds ratios for doubling of microRNA (miR) expression level

Table 1 – Baseline characteristics

	Total (n=263)
Demographics	
Age	67 (±13)
Male gender	189 (72)
Caucasian ethnicity	244 (94)
Clinical characteristics	
Body mass index kg/m^2	28 (±5)
Heart rate, bpm	67 (±12)
Systolic blood pressure, mmHg	122 (±20)
Diastolic blood pressure, mmHg	73 (±11)
Biomarker level	
NT-proBNP (pmol/L)	139.6 (51.9 – 272.9)
HsTNT (ng/L)	18.0 (9.6 – 33.2)
CRP (mg/L)	2.2 (0.9 – 4.8)
Features of heart failure	
Duration of heart failure, years	4.6 (1.7 – 9.9)
NYHA class I	75 (29)
NYHA class II	115 (44)
NYHA class III or IV	69 (27)
Left ventricular function	
Systolic dysfunction	250 (95)
HFPEF	13 (5)
LVEF*	32 (±10)
Etiology of heart failure	
Ischemic heart disease	117 (44)
Hypertension	34 (13)
Secondary to valvular heart disease	12 (5)
Cardiomyopathy	68 (26)
Dilated	49 (19)
Hypertrophic	12 (5)
Non compaction	4 (1)
Unclassified	3 (1)
Unknown	19 (7)
Other	13 (5)
Medical history	
Myocardial infarction	94 (36)
PCI	82 (31)
CABG	43 (16)
Valvular heart disease	136 (53)
Atrial fibrillation	105 (40)
Other arrhythmia	82 (32)
ICD	151 (59)
CRT	78 (30)
Pacemaker	38 (15)
CVA	41 (16)

Table 1 – Baseline characteristics (*continued*)

	Total (n=263)
Chronic renal failure	136 (53)
Diabetes Mellitus	81 (31)
Known hypercholesterolemia	93 (35)
Hypertension	120 (46)
Sleep apnea	26 (10)
Intoxications	
Alcohol consumption (>1 unit/day)	108 (42)
Smoking	185 (71)
Ever	186 (72)
Current	26 (10)
Medication use	
ACE-i	173 (67)
ARB	75 (29)
Aldosteron antagonist	178 (68)
Diuretic	237 (90)
Beta-blocker	232(88)
Aspirin	45 (17)
Vitamin K antagonist	200 (77)
Nitrates	44 (17)
Digoxin	59 (23)
Antiarrhythmics	39 (15)

Normally distributed continuous variables are presented as mean (± standard deviation). Non-normally distributed continuous variables are expressed as median (25^{th} – 75^{th} percentile). Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts, because of missing values. ACE-I = ace inhibitor; ARB = angiotensin II receptor blocker; CABG = coronary artery bypass grafting; COPD = chronic obstructive pulmonary disease; CRP = C-reactive protein; CRT = cardiac resynchronization therapy; CVA = cerebrovascular accident; HFPEF = heart failure with preserved ejection fraction; HsTNT = high sensitive cardiac troponin T; ICD = implantable cardioverter / defibrillator; LVEF = left ventricular ejection fraction; NT-proBNP = N-terminal pro-B-type natriuretic peptide NYHA = New York heart association; PCI = percutaneous coronary intervention.

* Baseline echocardiograms were available in 72% of all patients because of logistic reasons.

Clinical endpoints

The primary, composite endpoint was reached by 41 patients (16%), during a median follow-up of 0.9 [0.6-1.4] years: 5 patients died from a cardiovascular cause, 35 patients were re-hospitalized for worsened HF and 1 patient underwent heart transplantation. Of the 35 patients reaching the primary endpoint because of re-hospitalization for HF, 16 patients died eventually during further follow-up, of whom 12 died from a cardiovascular cause. Overall all-cause mortality was thus 21 (8.0%).

Of all baseline variables, NYHA class (HR 1.98; CI 1.24 – 3.16 for III/IV vs I/II), baseline NT-proBNP (HR per two-fold difference of baseline NT-proBNP 1.42; CI 1.09 – 1.86), and baseline CRP (HR per two-fold difference of baseline CRP 1.29; CI 1.05 – 1.60) were independently associated with the primary endpoint. As shown in table 2, none of the baseline miR values were associated with the primary endpoint, or the secondary endpoint comprising HF hospitalizations only.

Table 2 – Associations between baseline miRs and the primary endpoint and secondary endpoint

	Primary endpoint		Secondary endpoint	
	HR* (95% CI)	P	HR (95% CI)	P
miR-1254	1.01 (0.92–1.09)	0.91	1.00 (0.91–1.09)	0.99
miR-22-3p	1.02 (0.93–1.12)	0.61	1.00 (0.90–1.09)	0.85
miR-345-5p	0.99 (0.89–1.09)	0.81	0.95 (0.86–1.06)	0.38
miR-378a-3p	1.00 (0.91–1.11)	0.93	0.99 (0.89–1.09)	0.77
miR-423-5p	1.01 (0.91–1.11)	0.92	0.97 (0.87–1.07)	0.53
miR-486-5p	1.04 (0.94–1.15)	0.47	1.00 (0.89–1.11)	0.93
miR-320a	1.04 (0.95–1.13)	0.44	1.02 (0.93–1.12)	0.71

miR = microRNA.

Primary endpoint: cardiac death, cardiac transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened heart failure; secondary endpoint: hospitalization for the management of acute or worsened heart failure.

* Hazard ratios (HRs) and corresponding confidence intervals (CIs) were calculated using Cox regression analyses and are given per doubling of level of miR.

Temporal miR patterns and the primary endpoint

During follow-up, we collected 923 blood samples, of which 885 were drawn before the occurrence of the primary endpoint (median of 3 (IQR 2-5) samples per patient). MiRs were successfully determined in 873 of these samples. The temporal pattern of miR-22-3p was inversely associated with the primary endpoint (age and gender adjusted HR per doubling of miR-22-3p level, 0.64; CI 0.47 - 0.77; p<0.001), i.e. higher miR-22-3p was associated with lower risk of events (table 3). As described above, baseline measurements of several miRs were associated with ICM. On the other hand, NYHA class was independently associated with the primary endpoint. Therefore, the baseline variables ICM and NYHA class were added to the models. After adjustment for age, gender, NYHA class and ICM, the association between the temporal pattern of miR-22-3p and the primary endpoint remained present (HR per doubling of miR-22-3p level 0.71; CI 0.44 - 0.94; p=0.005). When serial NYHA class assessments were added to the model instead of baseline NYHA assessments only,

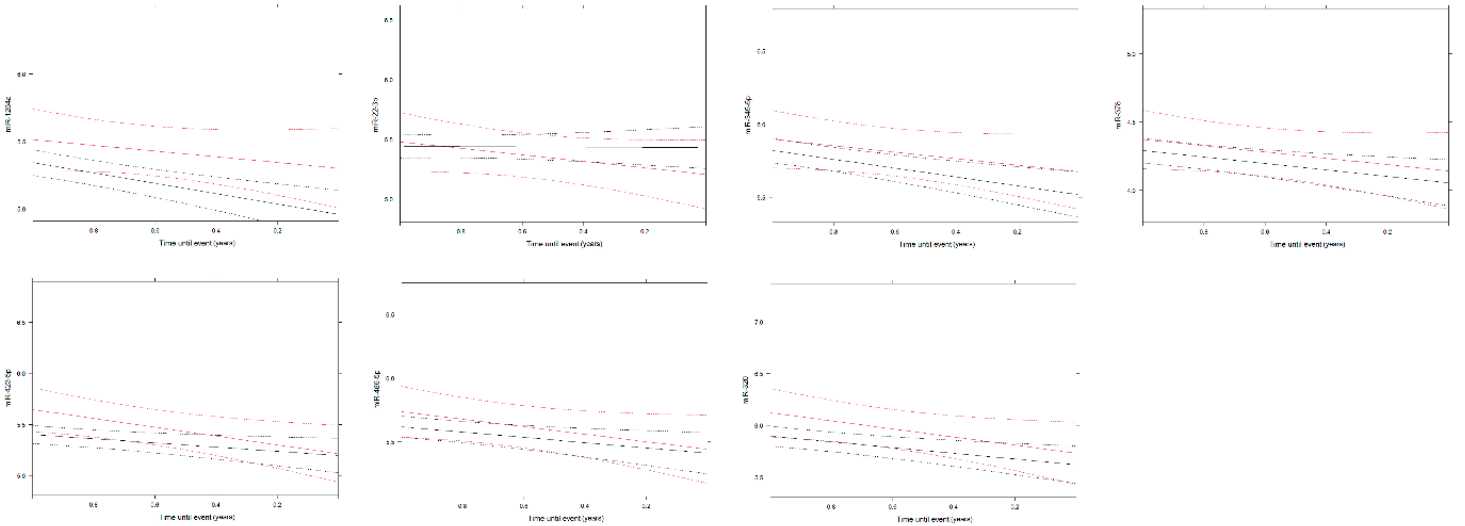


Figure 3 - Temporal evolution of microRNA levels

Temporal evolution was estimated by using mixed models. Y-axis: arbitrary units [a.u.] representing relative miRNA expression levels. X-axis: time in years as the endpoint approaches (time=0 denotes the moment that the endpoint occurs for the patients reaching the endpoint, and the moment of right-censoring for the patients not reaching the endpoint). Red solid line: miR expression level in patients reaching the endpoint. Red dotted lines: 95% confidence interval. Black solid line: miR expression level in patients not reaching the endpoint. Black dotted line: 95% confidence interval.

Table 3 - Associations between temporal miR patterns and the primary endpoint

	miR-1254		miR-22-3p		miR-345-5p		miR-378a-3p	
	HR* (95% CI)	p	HR* (95% CI)	p	HR* (95% CI)	p	HR* (95% CI)	p
Temporal pattern of miR value								
Adjusted for age and gender	1.02 (0.71–1.26)	0.782	0.64 (0.47–0.77)	<0.001	1.01 (0.97–1.04)	0.624	0.84 (0.69–1.03)	0.085
Multivariable adjusted [y]	0.97 (0.78–1.22)	0.757	0.71 (0.44–0.94)	0.005	1.00 (0.97–1.03)	0.921	0.88 (0.71–1.06)	0.148
Slope of temporal pattern								
Adjusted for age and gender	1.29 (0.75–2.49)	0.456	0.20 (0.09–0.50)	<0.001	NA	NA	NA	NA
Multivariable adjusted [y]	1.30 (0.79-2.35)	0.288	0.33 (0.20–0.51)	<0.001	NA	NA	NA	NA
Area under the curve of temporal pattern								
Adjusted for age and gender	1.09 (0.97–1.27)	0.206	1.00 (0.80–1.14)	0.898	NA	NA	NA	NA
Multivariable adjusted [y]	1.26 (0.95–1.53)	0.100	0.99 (0.88–0.17)	0.846	NA	NA	NA	NA

Table 3 - Associations between temporal miR patterns and the primary endpoint (continued)

	miR-423-5p		miR-486-5p		miR-320a	
	HR* (95% CI)	p	HR* (95% CI)	p	HR* (95% CI)	p
Temporal pattern of miR value						
Adjusted for age and gender	1.01 (0.97–1.05)	0.800	1.00 (1.00–1.01)	0.069	0.92 (0.74–1.19)	0.516
Multivariable adjusted [y]	1.00 (0.95–1.08)	0.845	1.00 0.99–1.01)	0.380	1.01 (0.78–1.22)	0.896
Slope of temporal pattern						
Adjusted for age and gender	NA	NA	NA	NA	0.51 (0.29–0.90)	0.012
Multivariable adjusted [y]	NA	NA	NA	NA	0.59 (0.33–1.12)	0.102
Area under the curve of temporal pattern						
Adjusted for age and gender	NA	NA	NA	NA	1.16 (0.94–1.42)	0.257
Multivariable adjusted [y]	NA	NA	NA	NA	1.19 (0.88–1.44)	0.330

miR = microRNA.

* Hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) are given per doubling of value, slope or area under the curve at any point in time.

[y] Adjusted for age, gender, New York Heart Association class and ischemic cardiomyopathy.

the association between miR miR-22-3p and the primary endpoint also remained present (HR per doubling of miR-22-3p level, 0.52; CI 0.44 - 0.61; p<0.001). The temporal patterns of the other 6 miRs were not significantly associated with the primary endpoint.

Baseline echocardiograms were available in 72% of all patients due to logistic reasons. After correction for left ventricular ejection fraction in this subgroup, miR-22-3p remained significantly associated with the primary endpoint (HR per doubling of miR-22-3p level, 0.91; CI 0.85 - 0.95; p<0.001).

Figure 3 shows that in the patients who reached the endpoint, miR-22-3p values declined as this endpoint approached, while in the patients who did not reach the endpoint, miR-22-3p values remained stable.

Slope and area under the curve of the temporal miR patterns and the primary endpoint

The hazard ratios for the doubling of the slopes and areas under the curve of the temporal miR patterns are displayed in table 3. For 4 of the miRs (miR-345-5p, miR-378a-3p, miR-423-5p, and miR-486-5p), slopes remained virtually constant over time, and hazard ratios were not calculable. The slope of the temporal pattern of miR-22-3p was inversely associated with the primary endpoint after multivariable adjustment (HR per doubling of miR-22-3p slope at any given time point, 0.33; CI 0.20–0.51; p<0.001), while the slopes of the patterns of miR-1254 and miR-320a were not. None of the areas under the curves of the temporal miR patterns were associated with the primary endpoint.

Temporal patterns of miRs, NT-proBNP, Hs-TNT and CRP combined in a multiple-marker model

After constructing the multivariable models that corrected for age, sex, ICM and NYHA class, we proceeded to additionally adjust the association between the temporal miR-22-3p pattern and the primary endpoint for temporal patterns of the other biomarkers which were measured in this study (NT-proBNP, HsTNT and CRP). We did this by entering the multivariably adjusted temporal pattern of miR-22-3p, as well as the multivariably adjusted temporal patterns of NT-proBNP, HsTNT and CRP simultaneously into one model. We found an independent association of miR-22-3p (HR per doubling of miR-22-3p level at any given time point, 0.61; CI 0.51–0.73; p<0.001) with the primary endpoint. Temporal patterns of NT-proBNP and CRP level were also independently associated with the primary endpoint in the

multiple-marker model (HRs: 1.34 (1.04–1.71) and 2.14 (1.59–2.88), respectively), while the temporal pattern of HsTNT was not (HR: 1.30 (0.91–1.84)). The slope of the temporal pattern of miR-22-3p also remained significantly associated with the primary endpoint after adjustment for the slopes of the temporal patterns of NT-proBNP, Troponin T and CRP (HR per doubling of miR-22-3p slope at any given time point, 0.55 (0.46–0.65); p<0.001).

Repeated miR measurements and other biomarkers

Associations between repeatedly measured miR and repeatedly measured NT-proBNP, HsTNT and CRP, calculated using generalised estimating equations, are displayed in table 4. Of note are the significant inverse associations between miR-345-5p and CRP (β-coefficient [95%CI]: -0.04 [-0.07 – -0.01] 10log(mg/L) per 10log increase in miR-345-5p level; p=0.012) and miR-22-3p and HsTNT (β-coefficient [95%CI]: -0.01 [-0.02 – -0.00] 10log(mg/L) per 10log increase in miR-22-3p level; p=0.014; as well as the borderline significant inverse association between miR-22-3p and CRP (β-coefficient [95%CI]: -0.02 [-0.04 – 0.00] 10log(mg/L) per 10log increase in miR-22-3p level; p=0.051). No further associations between repeatedly measured miR and repeatedly measured NT-proBNP, HsTNT and CRP were found.

Table 4 – Associations between repeatedly measured miRs and B-type natriuretic peptide, high sensitive cardiac troponin T and C- reactive protein

	NT-proBNP		HsTNT		CRP	
	Beta coefficient (95% CI)	P	Beta coefficient (95% CI)	P	Beta coefficient (95% CI)	P
miR-1254	0.07 (-0.05 – 0.02)	0.27	0.00 (-0.01 – 0.01)	0.59	0.00 (-0.02 – 0.02)	0.92
miR-22-3p	-0.01 (-0.022 – -0.00)	0.14	-0.01 (-0.02 – -0.00)	0.014	-0.03 (-0.06 – 0.00)	0.051
miR-345-5p	-0.04 (-0.02 – 0.01)	0.57	-0.01 (-0.02 – 0.01)	0.35	-0.04 (-0.07 – -0.01)	0.012
miR-378a-3p	-0.01 (-0.02 – 0.00)	0.12	0.00 (-0.01 – 0.01)	0.91	-0.02 (-0.04 – 0.01)	0.27
miR-423-5p	0.00 (-0.02 – 0.01)	0.81	-0.01 (-0.02 – 0.00)	0.17	-0.01 (-0.04 – 0.03)	0.63
miR-486-5p	-0.01 (-0.02 – 0.01)	0.43	-0.01 (-0.02 – 0.01)	0.37	-0.03 (-0.06 – 0.00)	0.070
miR-320a	0.00 (-0.01 – 0.02)	0.69	0.00 (-0.01 – 0.01)	0.74	0.01 (-0.02 – 0.03)	0.71

Beta coefficients and corresponding 95% confidence intervals (CIs) were calculated using generalised estimating equations with 10log transformed microRNAs (miRs) as independent variables and 10log transformed N-terminal pro-B-type natriuretic peptide (NT-proBNP), high sensitive cardiac troponin T (HsTNT) and C- reactive protein (CRP) as dependent variables.

DISCUSSION

In this prospective, observational cohort of CHF patients, temporal miR-22-3p patterns were inversely and independently associated with adverse outcome. The association was independent of temporal NT-proBNP, HsTNT and CRP patterns. The instant rate of change in miR-22-3p level (in terms of the slope of the temporal pattern) was also inversely associated with adverse outcome. Moreover, we found inverse associations of temporal patterns of miR-22-3p with temporal patterns of HsTNT and CRP. An additional finding of this study was the significant, inverse association between ICM and miR-1254, miR486-5p and miR-320a.

Our study has several strengths. Although the number of incident endpoints was limited, this is the largest study to date that has examined miRs in a prospective cohort of patients with chronic HF. NT-proBNP, HsTNT and CRP were measured concomitantly, and, in addition, frequent repeated measurements were performed during follow-up, both of the miRs and of the established blood biomarkers. Statistical models containing all these, repeatedly measured, biomarkers were constructed and modern statistical methods were applied ('joint modelling'), which take into account the continuous, dynamic changes in biomarker patterns and thus result in less bias than simpler analyses such as delta's between two measurements. Herewith, the study expands existing evidence with regard to miRs and cardiovascular disease in several respects.

MiR-22-3p has previously received attention in the cancer field; it has been implicated in the regulation of various cellular processes, including cell growth, apoptosis, motility, and the cell cycle.31 With regard to cardiovascular disease, fundamental experiments have demonstrated that expression of miR-22-3p is enriched in striated muscle tissues (i.e. cardiac and skeletal muscles); and miR-22-3p is thought to be required for normal cardiac remodeling in response to stresses.32 A recent study in miR-22 deficient mice demonstrated that miR-22 depleted hearts quickly progress to adverse cardiac remodeling and develop cardiac dilation, with increased cardiomyocyte loss and deposition of fibrotic tissue, and signs of heart failure.33 Another study also found that mice without miR-22 develop dilatation of myocardium more quickly.34 Interestingly, several animal models have shown that miR-22 may potentially serve as a therapeutic target in cardiac hypertrophy.33 Recently, Gupta et al found that pharmacological inhibition of miR-22 post-infarction activated cardiac autophagy, led to improved cardiac function, and inhibited cardiac remodeling in older mice.35 They also found that circulating miR-22 provides prognostic information in HF patients. Specifically, they demonstrated

an association between higher baseline levels of circulating miR-22 and mortality in154 HF patients. Repeated measurements were not performed. In the present study, although we saw a temporal decline of miR-22-3p levels in patients with adverse clinical outcome, we could not demonstrate a significant association between higher baseline miR-22-3p and clinical outcome. The shorter follow-up duration of our study (median of approx. 1 year, versus 3 years follow-up in the study of Gupta et al) may have contributed to this. Furthermore, in an earlier study, Goren et al, demonstrated that elevated serum levels of miR-22-3p identify patients with systolic heart failure using 30 stable chronic systolic heart failure patients and 30 controls.24 This seeming discrepancy with our study could possibly be explained by differences in study design. While Goren et al focused on diagnostic properties of miR-22-3p by comparing cases and controls, we focused on prognostic value of miR-22-3p in patients with established CHF. To the best of our knowledge, other cardiovascular studies on miR-22-3p in human subjects are lacking. Altogether, our study is in line with previous fundamental experiments which demonstrated that loss of miR-22-3p leads to dilatation of myocardium and progressive HF. Our study confirms that lower levels of miR-22-3p are associated with worsening of CHF, and that miR-22-3p may complement established HF biomarkers in prognostication.

Previously, several studies using animal models have shown decreased levels of miR-378a-3p during cardiac remodelling36 and in HF due to cardiac hypertrophy.37-39 Furthermore, a significant down-regulation of miR-378a-3p in hearts with dilated cardiomyopathy compared to non-failing control hearts has been observed.23 Since the presence of miR-378a-3p is required to resist ventricular remodeling during cardiac stress, it may offer therapeutic potential for the management of HF.38 It has been suggested, that up-regulation of miR-378a-3p could serve as a potential novel treatment for apoptosis and ischemic heart disease, since up-regulation of this miR enhanced cell viability, and inhibited apoptosis and necrosis in myocardium of rats.40 In the current study, we also observed a tendency towards an inverse association between the temporal pattern of miR378a-3p and the primary endpoint in univariable models. These results concur with the aforementioned studies.

Another miR that has previously been associated with HF, is miR-423-5p.19,21,22,24 However, in our study, there was no association between the temporal pattern of miR-423-5p and the primary endpoint. Existing studies on miR-423-5p in humans all compared single measurements in (C)HF patients with measurements in healthy controls, and imply that this miR has diagnostic value for HF.19,21,24 We

did not evaluate the diagnostic value of miRs, but the prognostic value of serial miR measurements in patients with known CHF. Although miR-423-5p may discriminate between HF patients and healthy controls, it may lack prognostic value in patients with longstanding CHF. Earlier findings support this hypothesis.20 Bauters et al serially measured serum levels of circulating miR-423-5p in 246 patients with a first anterior Q-wave myocardial infarction, at discharge and subsequently at 3 months and 1 year. They found no association between miR-423-5p levels and indices of left ventricular function and left ventricular remodeling, which were serially assessed during a 1-year period after the index myocardial infarction, nor with B-type natriuretic peptide. They concluded that miR-423-5p is not a useful biomarker of left ventricular remodeling after myocardial infarction.20

The selection of the miRs for the current investigation was based on previous studies, which used animal models and relatively small patient groups. Some of these studies have shown associations between miR-1254, miR-345-5p, miR-486-5p and miR-320a and heart failure.19,21,24,41,42 Again, these were mostly studies that compared HF patients to healthy controls. In our study, we did not find any associations between these miRs and poor outcome in CHF. Multiple other miRs, such as miR-133a,43 have previously been associated with HF.44 Serial measurements of these other miRs, which were not investigated in this study, warrant further investigation. This could lead to further improvements in CHF prognostication, as well as tailored adjustment of treatment.

Some aspects of this study warrant consideration. For the current investigation we only used the first round of inclusions of the ongoing Bio-SHiFT study (n=263). It should be noted that the full Bio-SHiFT cohort is designed to have sufficient statistical power to enable correction for multiple testing in case of application of modern, large-scale biomarker measurement methods. However, the current investigation examines only 7 miRs, which were chosen based on pathophysiological considerations. Thus, the current investigation was hypothesis-driven and not data-driven, rendering correction for multiple testing unnecessary in this particular case. Furthermore, with an average follow-up ~1 year, the number of primary endpoints in the current investigation (n=41) was limited, and consequently so was the number of covariables that could be entered into the models. The lack of a replication population is another limitation of the study. Finally, the majority of the patient population was in NYHA functional class I or II, and thus at relatively low risk. Accordingly, the primary outcome was predominantly composed of heart failure hospitalizations. This may have hampered the prognostic information of the

other miRs tested in this study. Future studies using high-risk patient populations should therefore re-investigate the prognostic information carried by repeated assessment of these miRs.

Remarkably, in the present study, the rate of HFPEF was very low compared to other epidemiological studies. This can most likely be attributed to the fact that in the Netherlands, most HFPEF patients are treated by the general practitioner or in secondary referral centres, while the current study was performed in two centres which were both tertiary referral centres. Potential inclusion bias is not a likely reason for the low HPEF rate, because consecutive patients were screened in both participating centres. Moreover, the number of patients on vitamin K antagonists was quite high in this study, most likely due to the high number of patients with left ventricular aneurysms and thrombi, as well as the high number of patients with atrial fibrillation.

In conclusion, in patients with stable CHF, the temporal pattern of circulating miR-22-3p is a strong and independent predictor of prognosis. Thus, the use of individual patterns of change of circulating miR-22-3p for CHF prognostication, as well as for tailored adjustment of treatment, warrants further investigation.

REFERENCES

1. Ponikowski P, Voors AA, Anker SD, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur.J.Heart Fail. 2016;18:891-975.
2. Yancy CW, Jessup M, Bozkurt B, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 2013;62:e147-e239.
3. Januzzi JL, Troughton R. Are serial BNP measurements useful in heart failure management? Serial natriuretic peptide measurements are useful in heart failure management. Circulation 2013;127:500-507.
4. de Couto G, Ouzounian M, and Liu PP. Early detection of myocardial dysfunction and heart failure. Nat Rev Cardiol 2010;7:334-344.
5. Savarese G, Trimarco B, Dellegrottaglie S, et al. Natriuretic peptide-guided therapy in chronic heart failure: a meta-analysis of 2,686 patients in 12 randomized trials. PLoS One. 2013;8:e58287.
6. Porapakkham P, Porapakkham P, Zimmet H, et al. B-type natriuretic peptide-guided heart failure therapy: A meta-analysis. Arch Intern Med 2010;170:507-514.
7. Miller WL, Hartman KA, Burritt MF, et al. Serial biomarker measurements in ambulatory patients with chronic heart failure: the importance of change over time. Circulation 2007;116:249-257.
8. Yan RT, White M, Yan AT, et al. Usefulness of temporal changes in neurohormones as markers of ventricular remodeling and prognosis in patients with left ventricular systolic dysfunction and heart failure receiving either candesartan or enalapril or both. Am J Cardiol 2005;96:698-704.
9. Troughton R, Michael FG, and Januzzi JL, Jr. Natriuretic peptide-guided heart failure management. Eur Heart J 2014;35:16-24.
10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-297.
11. Gilad S, Meiri E, Yogev Y, et al. Serum microRNAs are promising novel biomarkers. PLoS One. 2008;3:e3148.
12. Chen JF, Murchison EP, Tang R, et al. Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proc Natl Acad Sci U.S A 2008;105:2111-2116.
13. Costa Martins PA, Bourajjaj M, Gladka M, et al. Conditional dicer gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling. Circulation 2008;118:1567-1576.
14. Creemers EE, Tijsen AJ, and Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res 2012;110:483-495.
15. Vogel B, Keller A, Frese KS, et al. Refining diagnostic microRNA signatures by whole-miRNome kinetic analysis in acute myocardial infarction. Clin Chem 2013;59:410-418.
16. Zampetaki A, Willeit P, Tilling L, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. J Am Coll Cardiol 2012;60:290-299.

17. Corsten MF, Dennert R, Jochems S, et al. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. Circ Cardiovasc Genet. 2010;3:499-506.
18. Olivieri F, Antonicelli R, Lorenzi M, et al. Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. Int J Cardiol 2013;167:531-536.
19. Goldraich LA, Martinelli NC, Matte U, et al. Transcoronary gradient of plasma microRNA 423-5p in heart failure: evidence of altered myocardial expression. Biomarkers 2014;19:135-141.
20. Bauters C, Kumarswamy R, Holzmann A, et al. Circulating miR-133a and miR-423-5p fail as biomarkers for left ventricular remodeling after myocardial infarction. Int J Cardiol 2013;168:1837-1840.
21. Tijsen AJ, Creemers EE, Moerland PD, et al. MiR423-5p as a circulating biomarker for heart failure. Circ Res 2010;106:1035-1039.
22. Dickinson BA, Semus HM, Montgomery RL, et al. Plasma microRNAs serve as biomarkers of therapeutic efficacy and disease progression in hypertension-induced heart failure. Eur J Heart Fail. 2013;15:650-659.
23. Naga Prasad SV, Duan ZH, Gupta MK, et al. Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks. J Biol Chem 2009;284:27487-27499.
24. Goren Y, Kushnir M, Zafrir B, et al. Serum levels of microRNAs in patients with heart failure. Eur J Heart Fail. 2012;14:147-154.
25. McMurray JJ, Adamopoulos S, Anker SD, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 2012;33:1787-1847.
26. Dickstein K, Cohen-Solal A, Filippatos G, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur Heart J 2008;29:2388-2442.
27. Paulus WJ, Tschope C, Sanderson JE, et al. How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. Eur Heart J 2007;28:2539-2550.
28. Rao PK, Toyama Y, Chiang HR, et al. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res 2009;105:585-594.
29. Rizopoulos D. JM: An R Package for the Joint Modelling of Longitudinal and Time-to-Event Data. Journal of Statistical Software 2010;35:1-33.
30. Rizopoulos D. Joint Models for Longitudinal and Time-to-Event Data: With Applications in R. 2012.
31. Li J, Liang S, Yu H, et al. An inhibitory effect of miR-22 on cell migration and invasion in ovarian cancer. Gynecol.Oncol. 2010;119:543-548.

32. Huang ZP, Wang DZ. miR-22 in cardiac remodeling and disease. Trends Cardiovasc. Med. 2014;24:267-272.
33. Huang ZP, Chen J, Seok HY, et al. MicroRNA-22 regulates cardiac hypertrophy and remodeling in response to stress. Circ.Res. 2013;112:1234-1243.
34. Gurha P, Abreu-Goodger C, Wang T, et al. Targeted deletion of microRNA-22 promotes stress-induced cardiac dilation and contractile dysfunction. Circulation 2012;125:2751-2761.
35. Gupta SK, Foinquinos A, Thum S, et al. Preclinical Development of a MicroRNA-Based Therapy for Elderly Patients With Myocardial Infarction. J.Am.Coll.Cardiol. 2016;68:1557-1571.
36. Knezevic I, Patel A, Sundaresan NR, et al. A novel cardiomyocyte-enriched microRNA, miR-378, targets insulin-like growth factor 1 receptor: implications in postnatal cardiac remodeling and cell survival. J Biol Chem 2012;287:12913-12926.
37. Nagalingam RS, Sundaresan NR, Gupta MP, et al. A cardiac-enriched microRNA, miR-378, blocks cardiac hypertrophy by targeting Ras signaling. J Biol Chem 2013;288:11216-11232.
38. Nagalingam RS, Sundaresan NR, Noor M, et al. Deficiency of Cardiomyocyte-specific MicroRNA-378 Contributes to the Development of Cardiac Fibrosis Involving a Transforming Growth Factor beta (TGFbeta1)-dependent Paracrine Mechanism. J Biol Chem 2014;289:27199-27214.
39. Ganesan J, Ramanujam D, Sassi Y, et al. MiR-378 controls cardiac hypertrophy by combined repression of mitogen-activated protein kinase pathway factors. Circulation 2013;127:2097-2106.
40. Fang J, Song XW, Tian J, et al. Overexpression of microRNA-378 attenuates ischemia-induced apoptosis by inhibiting caspase-3 expression in cardiac myocytes. Apoptosis. 2012;17:410-423.
41. Li X, Zhang X, Wang T, et al. Regulation by bisoprolol for cardiac microRNA expression in a rat volume-overload heart failure model. J Nanosci.Nanotechnol. 2013;13:5267-5275.
42. Small EM, O'Rourke JR, Moresi V, et al. Regulation of PI3-kinase/Akt signaling by muscle-enriched microRNA-486. Proc Natl Acad Sci U.S A 2010;107:4218-4223.
43. Besler C, Urban D, Watzka S, et al. Endomyocardial miR-133a levels correlate with myocardial inflammation, improved left ventricular function, and clinical outcome in patients with inflammatory cardiomyopathy. Eur.J.Heart Fail. 2016.
44. Vegter EL, van der MP, De Windt LJ, et al. MicroRNAs in heart failure: from biomarker to target for therapy. Eur.J.Heart Fail. 2016;18:457-468.

Supplemental table – Associations between baseline characteristics and miRNAs measured at baseline

	miR1254a		miR22-3p		miR345-5p		miR378	
	Beta coefficient (95% CI)	P	Beta coefficient (95% CI)	P	Beta coefficient (95% CI)	P	Beta coefficient (95% CI)	P
Age*	0.02 (-0.03 – 0.08)	0.44	-0.04 (-0.09 – 0.01)	0.16	0.00 (-0.04 – 0.05)	0.88	-0.02 (-0.07 – 0,03)	0.34
Male gender	-0,10 (-0.40 – 0.21)	0.53	0.11 (-0.19 – 0.41)	0.46	0.01 (-0.25 – 0.27)	0.92	0.11 (-0.17 – 0.40)	0.42
BMI	0.00 (-0.03 – 0.03)	0.82	0.02 (-0.01 – 0.05)	0.12	0.01 (-0.02 – 0.03)	0.45	0.01 (-0.02 – 0.04)	0.49
HR*	0.05 (-0.01 – 0.11)	0.10	0.00 (-0.06 – 0.06)	0.93	0.02 (-0.03 – 0.07)	0.45	0.03 (-0.03 – 0.08)	0.37
SBP*	-0.01 (-0.05 – 0.02)	0.44	-0.01 (-0.04 – 0.03)	0.62	-0.01 (-0.04 – 0.01)	0.33	-0.02 (-0.06 – 0.01)	0.14
DBP*	-0.01 (-0.08 – 0.06)	0.79	0.00 (-0.07 – 0.06)	0.91	0.00 (-0.06 – 0.05)	0.96	0.01 (-0.05 – 0.07)	0.71
NYHA III/V	-0.10 (-0.41 – 0.21)	0.54	-0.27 (-0.57 – 0.04)	0.086	-0.14 (-0.40 – 0.13)	0.32	-0.24 (-0.53 – 0.04)	0.093
ICMP	-0.34 (-0.61 – -0.06)	0.017	-0.05 -0.32 – 0.22)	0.73	-21 (-0.44 – 0.03)	0.085	-0.23 (-0.49 – 0.02)	0.070
HCMP	0.31 (-0.09 – 0.72)	0.13	-0.25 (-0.65 – 0.14)	0.21	-0.03 (-0.38 – 0.32)	0.86	0.01 (-0.36 – 0.39)	0.95
Renal failure	0.12 (-0.16 – 0.40	0.39	-0.13 (-0.40 – 0.14)	0.34	-0.12 (-0.37 – 0.11)	0.30	-0.10 (-0.36 – 0.16)	0.45
DM	0.00 (-0.30 – 0.29)	0.97	-0.01 (-0.31 – 0.28)	0.93	-0.04 (-0.29 – 0.22)	0.76	0.03 (-0.24 – 0.31)	0.82
HT	0.29 (0.02 – 0.57)	0.038	-0.02 (-0.29 – 0.25)	0.90	0.09 (-0.15 – 0.33)	0.47	0.08 (-0.18 – 0.34)	0.54
Ever-smoker	0.13 (-0.14 – 0.44)	0.44	-0.16 (-0.46 – 0.14)	0.29	-0.06 (-0.31 –0.20)	0.67	-0.04 (-0.32 – 0.25)	0.80

Beta coefficients and corresponding 95 % confidence intervals (CIs) were calculated using linear regression with the 10log transformed microRNAs (MIRs) as dependent variables.

BMI = body mass index; COPD = chronic obstructive pulmonary disease; DBP = diastolic blood pressure; DM = diabetes mellitus; HCMP = hypertensive cardiomyopathy; HR = heart rate; HT = hypertension; ICMP = ischemic cardiomyopathy; NYHA = New York heart association class; SBP = systolic blood pressure;

* Per 5 points increase

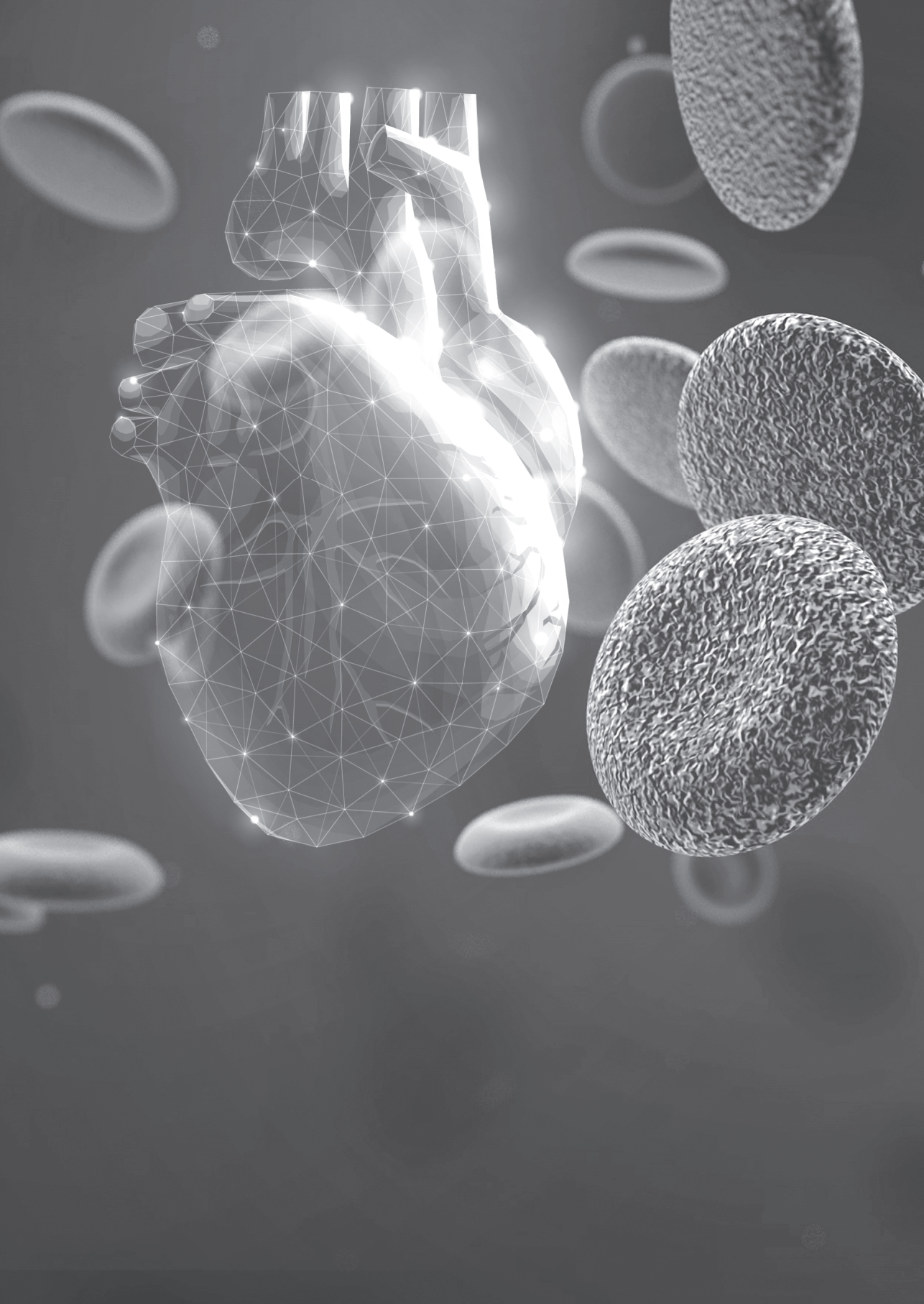
Supplemental table – Associations between baseline characteristics and miRNAs measured at baseline (continued)

	miR423-5p		miR486-5p		miR320	
	Beta coefficient (95% CI)	P	Beta coefficient (95% CI)	P	Beta coefficient (95% CI)	P
Age*	-0.03 (-0.08 – 0.02)	0.31	-0.02 (-0.06 – 0.03)	0.52	0.00 (-0.04 – 0.06)	0.87
Male gender	0.12 (-0.17 – 0.40)	0.42	0.13 (-0.14 – 0.39)	0.35	0.13 (-0.18 – 0.43)	0.41
BMI	0.00 (-0.03 – 0.03)	0.92	0.01 (-0.02 – 0.03)	0.44	0.00 (-0.02 – 0.03)	0.76
HR*	0.02 (-0.04 – 0.08)	0.48	0.03 (-0.02 – 0.09)	0.22	0.03 (-0.03 – 0.09)	0.30
SBP*	-0.02 (-0.05 – 0.01)	0.30	-0.02 (-0.05 – 0.01)	0.28	-0.01 (-0.05 – 0.02)	0.42
DBP*	-0.01 (-0.07 – 0.05)	0.64	0.00 (-0.06 – 0.06)	0.96	-0.01 (-0.08 – 0.05)	0.68
NYHA III/V	-0.09 (-0.38 – 0.20)	0.54	-0.09 (-0.36 – 0.18)	0.52	-0.02 (-0.33 –0.29)	0.91
ICMP	-0.21 (-0.46 – 0.05)	0.11	-0.26 (-0.50 – -0.02)	0.035	-0.32 (-0.60 – -0.05)	0.021
HCMP	-0.10 (-0.47 – 0.28)	0.61	0.17 (-0.18 – 0.53)	0.33	0.17 (-0.23 – 0.57)	0.41
Renal failure	-0.05 (-0.31 – 0.20)	0.69	-0.13 (-0.37 – 0.11)	0.30	0.15 (-0.13 – 0.43)	0.30
DM	-0.03 (-0.30 – 0.25)	0.84	0.03 (-0.23 – 0.29)	0.84	-0.11 (-0.41 – 0.19)	0.49
HT	0.08 (-0.17 – 0.34)	0.53	0.00 (-0.24 – 0.24)	0.98	0.12 (-0.16 –0.39)	0.42
Ever-smoker	-0.07 (-0.35 – 0.22)	0.65	0.00 (-0.27 – 0.27)	0.99	0.08 (-0.22 – 0.39)	0.60

Beta coefficients and corresponding 95 % confidence intervals (CIs) were calculated using linear regression with the 10log transformed microRNAs (MIRs) as dependent variables.

BMI = body mass index; COPD = chronic obstructive pulmonary disease; DBP = diastolic blood pressure; DM = diabetes mellitus; HCMP = hypertensive cardiomyopathy; HR = heart rate; HT = hypertension; ICMP = ischemic cardiomyopathy; NYHA = New York heart association class; SBP = systolic blood pressure;

* Per 5 points increase



11

Patient-specific evolution of renal function in chronic heart failure patients dynamically predicts clinical outcome in the Bio-SHiFT study

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Patient-specific evolution of renal function in chronic heart failure patients dynamically predicts clinical outcome in the Bio-SHiFT study

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ABSTRACT

Renal dysfunction is an important component of chronic heart failure (CHF), but its single assessment does not sufficiently reflect clinically silent progression of CHF prior to adverse clinical outcome. Therefore, we aimed to investigate temporal evolutions of glomerular and tubular markers in 263 stable patients with CHF, and to det16ermine if their patient-specific evolutions during this clinically silent period can dynamically predict clinical outcome. We determined the risk of clinical outcome (composite endpoint of Heart Failure hospitalization, cardiac death, Left Ventricular Assist Device placement, and heart transplantation) in relation to marker levels, slopes and areas under their trajectories. In each patient, the trajectories were estimated using repeatedly measured glomerular markers: creatinine/estimated glomerular filtration rate (eGFR), cystatin C (CysC), and tubular markers: urinary N-acetyl-beta-D-glucosaminidase (NAG) and kidney injury molecule (KIM)-1, plasma and urinary neutrophil gelatinase-associated lipocalin (NGAL). During 2.2 years of follow-up, we collected on average 8 urine and 9 plasma samples per patient. All glomerular markers predicted the endpoint (univariable hazard ratio [95% confidence interval] per 20% increase: creatinine: 1.18 [1.07–1.31], CysC: 2.41[1.81–3.41], and per 20% eGFR decrease: 1.13[1.05–1.23]). Tubular markers, NAG, and KIM-1 also predicted the endpoint (NAG: 1.06[1.01–1.11] and KIM-1: 1.08[1.04–1.11]). Larger slopes were the strongest predictors (creatinine: 1.57[1.39–1.84], CysC: 1.76 [1.52–2.09], eGFR: 1.59[1.37–1.90], NAG: 1.26[1.11–1.44], and KIM-1: 1.64[1.38–2.05]). Associations persisted after multivariable adjustment for clinical characteristics. Thus, during clinically silent progression of CHF,

glomerular and tubular functions deteriorate, but not simultaneously. Hence, patient-specific evolutions of these renal markers dynamically predict clinical outcome in patients with CHF.

INTRODUCTION

Heart failure (HF) is the leading cause of hospitalization worldwide.[1] Despite declines in HF-related mortality as a result of current therapies, re-hospitalization rates for decompensation of chronic heart failure (CHF) remain high.[1,2] Several blood biomarkers that predict re-hospitalization and mortality have been identified in patients with CHF.[3] Still their predictive capabilities in practice are limited, and adequate risk assessment remains a challenge.[3] Estimation of renal dysfunction, which coexists and interacts with HF,[3] may improve risk stratification. Baseline glomerular dysfunction, as assessed by estimated glomerular filtration rate (eGFR), entails an unfavorable prognosis in CHF.[4–6] Besides glomerular impairment, such patients often have tubular damage due to tubulo-interstitial injury by renal tissue hypoperfusion or due to damaged glomerular barrier.[7,8] Notably, a single assessment of damaged tubules predicts adverse outcome in CHF independently of eGFR.[9–11]

It is clear that both glomerular and tubular function are important in patients with CHF, but their single assessment does not sufficiently reflect deterioration along the cardiorenal axis that occurs over time preceding adverse events. Yet the temporal evolution of renal function preceding the event may dynamically ascertain the clinically silent progression of the disease. Specifically, it would enable accurate investigation of whether, and to which degree, increasing (or decreasing) levels of renal biomarkers contribute to the patient's risk, regardless of whether these levels exceed established cut points at study baseline (i.e., a random point in time prior to event).

In the context of cardio-renal interplay, patients with CHF also display large biological heterogeneity. Renal function not only changes dynamically within a patient over time, but also differs from patient to patient. Hence, the true potential of renal markers in ascertaining individual disease progression and their accurate relation with clinical outcome can only be revealed if their patient-specific evolutions are considered. However, detailed individual temporal evolutions of renal function in CHF have never been described.

To overcome these issues, our aim was 2-fold: (i) to investigate the average (population) temporal evolutions of glomerular function (measured with plasma creatinine

[Cr], eGFR, and cystatin C [CysC]) and tubular status (measured with urinary kidney injury molecule [KIM]-1, N-acetyl-beta- D-glucosaminidase [NAG], and urinary and plasma neutrophil gelatinase-associated lipocalin [NGAL]) in stable patients with CHF; and (ii) to determine whether patient-specific (individual) evolutions of these renal biomarkers during a clinically silent period can dynamically predict clinical outcome. For this purpose we examined several aspects of the temporal evolution of each renal biomarker that may be relevant for clinical prediction.

RESULTS

Baseline characteristics

Table 1 displays the baseline characteristics. At baseline, patients who later experienced the endpoint were older; more frequently had diabetes and atrial fibrillation; had lower systolic blood pressure, higher New York Heart Association (NYHA) class, higher levels of N-terminal prohormone of brain natriuretic peptide (NT-proBNP), cardiac troponin T, CysC, urinary N-acetyl-beta-D-glucosaminidase (NAG), and plasma urinary neutrophil-gelatinase-associated-lipocalin (NGAL); and were more frequently on diuretics than the patients who remained endpoint-free.

Follow-up and study endpoints

From 263 patients with CHF, a total of 1912 urine and 1984 blood samples were collected with a median (interquartile range, IQR) of 8 (5–10) urine and 9 (5–10) plasma samples per patient. During a median (IQR) follow-up of 2.2 (1.4–2.5) years, 70 (27%) patients reached the primary endpoint: 56 patients were re-hospitalized for acute or worsened HF, 3 patients underwent heart transplantation, 2 patients underwent left ventricle assist device (LVAD) placement, and 9 patients died of cardiovascular causes.

Temporal evolution of glomerular function

Creatinine and eGFR. In patients who reached the composite endpoint, Cr levels on average showed an increasing pattern over time preceding the endpoint. In endpoint-free patients Cr levels were lower and remained stable during follow-up (Figure 1a). eGFR displayed similar dynamics (Figure 1b). Independently of baseline levels, repeatedly measured Cr and eGFR predicted the endpoint (per 20% increase of Cr levels: hazard ratio [95% confidence interval] 1.18 [1.07–1.31], $P = 0.004$, and

Table 1 | Patient characteristics in relation to the occurrence of the composite endpoint

Variable	Total	Composite endpoint reached		*P* value
		Yes	No	
N (%)	263 (100)	70 (27)	193 (73)	
Demographics				
Age, yr (mean ± SD)	67 ± 13	69 ± 13	66 ± 12	0.05
Men, *n* (%)	189 (72)	53 (76)	136 (70)	0.41
Clinical characteristics				
BMI, kg/m^2 (mean ± SD)	27.5 ± 4.7	27.6 ± 4.8	27.4 ± 4.7	0.80
Heart rate, bpm (mean ± SD)	67 ± 12	69 ± 13	67 ± 11	0.31
SBP, mm Hg (mean ± SD)	122 ± 20	117 ± 17	124 ± 21	0.02
DBP, mm Hg (mean ± SD)	72 ± 11	70 ± 10	73 ± 11	0.06
Features of heart failure				
NYHA class III or IV, n (%)	69 (26)	31 (44)	38 (20)	<0.001
HF-rEF n (%)	250 (95)	66 (94)	184 (95)	0.75
HF-pEF n (%)	13 (5)	4 (6)	9 (5)	
LVEF, % (mean ± SD)	32 ± 11	30 ± 11	33 ± 10	0.18
NT pro-BNP (pmol/l)*	137.3 (51.7–272.6)	282.4 (176.4–517.4)	95.3 (31.72–207.7)	<0.001
Hs-TnT (ng/l)*	18.0 (9.5–33.2)	31.9 (20.6–49.7)	13.9 (8.4–26.7)	<0.001
Etiology of heart failure, n (%)				
Ischemic	117 (44)	36 (51)	81 (42)	0.17
Hypertension	34 (13)	10 (14)	24 (12)	0.70
Secondary to valvular disease	12 (5)	5 (7)	7 (4)	0.23
Cardiomyopathy	68 (26)	15 (21)	53 (28)	0.32
Unknown or Others	32 (12)	4 (6)	28 (15)	
Medical history, n (%)				
Prior MI	96 (36)	32 (46)	64 (33)	0.06
Prior PCI	82 (31)	27 (39)	55 (28)	0.12
Prior CABG	43 (16)	13 (19)	30 (15)	0.57
Atrial fibrillation	106 (40)	36 (51)	70 (36)	0.03
Diabetes	81 (31)	32 (46)	49 (25)	0.002
Hypercholesterolemia	96 (36)	30 (43)	66 (34)	0.20
Hypertension	120 (46)	38 (54)	82 (42)	0.09
COPD	31 (12)	12 (17)	19 (10)	0.10
Medication use, n (%)				
Beta-blocker	236 (90)	61 (87)	175 (91)	0.40
ACE-I or ARB	245 (93)	63 (90)	182 (94)	0.22
Diuretics	237 (90)	68 (97)	169 (88)	0.02
Loop diuretics	236 (90)	68 (97)	168 (87)	0.02
Thiazides	7 (3)	3 (4)	4 (2)	0.28
Aldosterone antagonist	179 (68)	53 (76)	126 (65)	0.11

Table 1 | Patient characteristics in relation to the occurrence of the composite endpoint (*continued*)

Variable	Total	Composite endpoint reached		*P* value
		Yes	No	
Glomerular function markers*				
Creatinine, mg/dl	1.18 (0.99–1.49)	1.30 (1.02–1.52)	1.17 (0.98–1.45)	0.18
eGFR, ml/min per 1.73m^2	58 (43–76)	53 (40–73)	59 (44–77)	0.16
Cystatin C, mg/l	0.73 (0.57–0.97)	0.87 (0.71–1.03)	0.70 (0.53–0.90)	<0.001
KDOQI classification, n (%)				
eGFR ≥ 90 ml/min per 1.73m^2	28 (11)	7 (10)	21 (11)	0.18
eGFR 60–89 ml/min per 1.73m^2	95 (36)	20 (28)	75 (39)	
eGFR 30–59 ml/min per 1.73m^2	119 (45)	37 (53)	82 (42)	
eGFR < 30 ml/min per 1.73m^2	21 (8)	6 (9)	15 (8)	
Tubular markers*				
NAG, U/gCr (urine)	5.9 (3.8–9.3)	8.0 (6.0–11.0)	5.1 (3.3–8.0)	<0.001
KIM-1, ng/gCr (urine)	477.2 (247.0–938.6)	589.0 (255.0–957.2)	465.1 (237.6–911.5)	0.10
NGAL, µg/gCr (urine)	17.4 (9.2–32.6)	18.2 (10.0–50.5)	17.4 (9.0–31.4)	0.20
NGAL, ng/ml (plasma)	190.1 (133.5–280.0)	260.8 (169.5–355.4)	179.2 (127.9–244.5)	<0.001

ACE-I, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; BMI, body mass index; bpm, beats per minute; CABG, coronary artery bypass grafting; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HF-pEF, heart failure with preserved ejection fraction; HF-rEF, heart failure with reduced ejection fraction; LVEF, left ventricular ejection fraction; MI, myocardial infarction; NYHA, New York Heart Association; PCI, percutaneous coronary intervention; SBP, systolic blood pressure; TIA, transitory ischemic attack.

Normally distributed continuous variables are presented as mean ± SD, and non-normally distributed variables as median and interquartile range. Categorical variables are presented as numbers and percentages.

*All biomarkers levels were presented as median (interquartile range).

per 20% eGFR decrease: 1.13 [1.05–1.1.23], $P = 0.002$) (Table 2). Similarly, their larger slopes and larger area under the curve of the marker's trajectory (AUCm) predicted the endpoint (per 20% increase of Cr slope: 1.57 [1.39–1.84], $P < 0.001$, per 20% decrease of eGFR slope: 1.59 [1.37–1.90], $P < 0.001$) (per 20% increase of Cr's AUCm: 1.10 [1.03–1.18], $P = 0.010$, and eGFR's AUCm: 1.07 [1.02–1.11], $P < 0.001$). These risk estimates remained significant even after adjustment for clinical characteristics and dose changes of HF medications during followup. After adjustment for cardiac markers, Cr's levels and AUCm lost precision, whereas eGFR remained significant (Table 2). Table S1 shows similar results for HF hospitalizations (secondary endpoint).

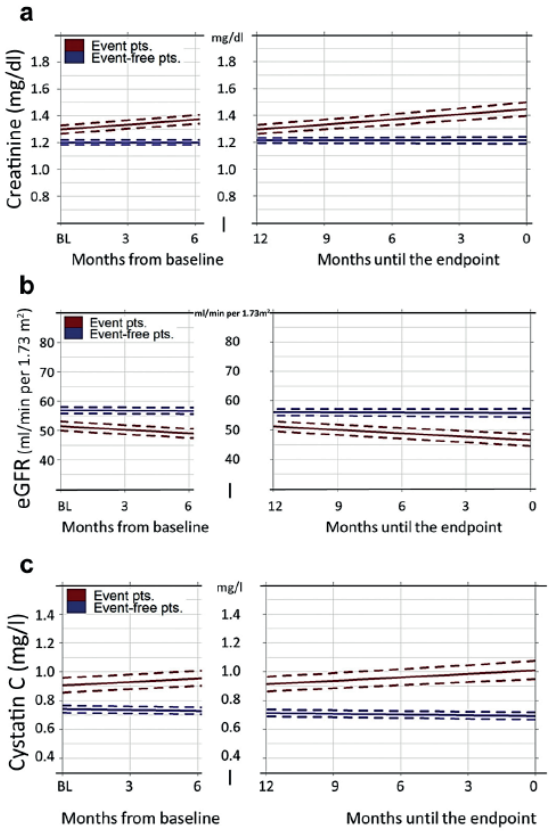


Figure 1 | Average evolution of glomerular function markers during follow-up. Average evolution in patients who reached the study endpoint (solid red line), and in endpoint-free patients (solid blue line). Dashed lines represent the 95% confidence interval. X-axis depicts the time from baseline (left part of the x-axis), and time remaining to the event (patients who experienced incident events) or last sample moment (patients who remained event-free; right part of the x-axis). Biomarker levels are presented on the y-axis. (a) Creatinine (mg/dl). (b) eGFR (ml/min per 1.73 m^2). (c) Cystatin C (µg/ml). BL, baseline; pts., patients.

Cystatin C. In patients who reached the composite endpoint, CysC showed on average higher baseline levels that increased further as the endpoint approached. In endpoint-free patients, CysC levels were lower and slightly decreased during follow-up (Figure 1c). Independently of baseline levels, CysC levels at any time during follow-up were associated with the endpoint (per 20% increase of CysC levels: 2.41 [1.81–3.41], $P < 0.001$) (Table 2). Similarly, larger slope and larger AUCm predicted the endpoint (1.76 [1.52–2.09], $P < 0.001$ and 1.32 [1.17–1.54], $P < 0.001$). These risk estimates remained significant after multivariable adjustments (Table 2). Supplementary Table S1 shows similar results for HF hospitalizations.

Table 2 | Associations between glomerular function markers and the composite endpoint

	Creatinine		eGFR		Cystatin C	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Baseline level[a]						
Model A	1.04 (0.99–1.09)	0.14	1.03 (0.99–1.07)	0.13	1.09 (1.05–1.14)	<0.001
Model B	1.02 (0.97–1.07)	0.49	1.02 (0.97–1.06)	0.48	1.07 (1.02–1.12)	0.007
Model C	0.98 (0.93–1.03)	0.46	0.98 (0.94–1.02)	0.28	1.00 (0.95–1.06)	0.89
Temporal evolution[b]						
Repeatedly measured levels						
Model 1	1.18 (1.07–1.31)	0.004	1.13 (1.05–1.23)	0.002	2.41 (1.81–3.41)	<0.001
Model 2	1.12 (1.02–1.23)	0.022	1.12 (1.06–1.20)	<0.001	2.16 (1.44–3.72)	<0.001
Model 3	1.05 (0.96–1.15)	0.28	1.09 (1.04–1.14)	<0.001	1.63 (1.35–2.30)	<0.001
Model 4	1.15 (1.08–1.24)	<0.001	1.10 (1.04–1.16)	<0.001	2.27 (1.99–2.59)	<0.001
Annual slope						
Model 1	1.57 (1.39–1.84)	<0.001	1.59 (1.37–1.90)	<0.001	1.76 (1.52–2.09)	<0.001
Model 2	1.65 (1.40–1.98)	<0.001	1.64 (1.38–2.02)	<0.001	2.00 (1.66–2.51)	<0.001
Model 3	1.37 (1.22–1.57)	<0.001	1.30 (1.16–1.46)	0.002	1.47 (1.32–1.66)	<0.001
Model 4	1.28 (1.16–1.43)	<0.001	1.18 (1.07–1.31)	0.001	1.63 (1.50–1.77)	<0.001
AUCm						
Model 1	1.10 (1.03–1.18)	0.010	1.07 (1.02–1.11)	<0.001	1.32 (1.17–1.54)	<0.001
Model 2	1.08 (1.01–1.15)	0.020	1.07 (1.02–1.12)	<0.001	1.23 (1.13–1.36)	<0.001
Model 3	1.04 (0.98–1.10)	0.17	1.06 (1.02–1.10)	<0.001	1.17 (1.08–1.28)	<0.001

AUCm, area under the curve of marker's trajectory; CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; LME, linear mixed effects.

[a]HRs and 95% CIs are given per 20% increase of creatinine and cystatin C, and 20% eGFR decrease. Model A is unadjusted. Model B is adjusted for age, sex, diabetes, atrial fibrillation, baseline New York Heart Association class, diuretics, and systolic blood pressure. Model C is adjusted for baseline NT-proBNP and hs-cTnT.

[b]HRs and 95% CIs are given per 20% increase of the level, slope, and AUCm of creatinine and cystatin C, and 20% decrease of the level, slope, and AUCm of eGFR. Model 1 is Cox model–adjusted for marker's baseline levels and LME model–adjusted for sampling time. Model 2 is Cox and LME model–adjusted for the clinical variables age, sex, diabetes, atrial fibrillation, baseline New York Heart Association class, diuretics, systolic blood pressure, and sampling time (LME). Model 3 is Cox and LME model–adjusted for baseline NT-proBNP and hs-cTnT and sampling time (LME). Model 4 is time-dependent Cox–adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.

Temporal evolution of tubular function

Overall, we found substantial associations between NAG, KIM-1, and NGAL, but only mild associations between these tubular markers and glomerular function markers (namely CysC), when assessed during follow-up (Table S2).

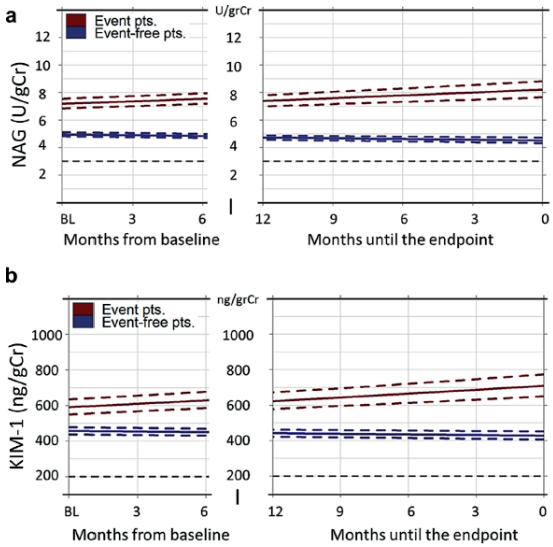


Figure 2 | Average evolution of tubular markers urinary NAG and KIM-1 during follow-up. For description see Figure 1. Dashed black lines represent the biomarkers' reference values. (a) Urinary N-acetyl-beta-D-glucosaminidase (NAG) (U/gCr). (b) Urinary kidney injury molecule-1 (KIM-1) (ng/gCr). BL, baseline; pts., patients.

Urinary NAG. In patients who reached the composite endpoint, NAG showed on average higher baseline levels that increased further as the endpoint approached. In endpoint-free patients, NAG levels were lower and decreased during follow-up (Figure 2a). Independently of baseline levels, higher NAG levels at any time during follow-up were associated with the endpoint (per 20% increase of NAG levels: 1.06 [1.01–1.11], P = 0.018). Similarly, larger NAG slope predicted the endpoint (1.26 [1.11–1.44], P = 0.004).These risk estimates remained significant after multivariable adjustments, except for NAG slope that became insignificant after controlling for cardiac markers (Table 3). Table S3 shows similar results for HF hospitalizations, except for NAG levels that lost significance after adjusting for cardiac markers.

Urinary KIM-1. In patients who reached the composite endpoint, KIM-1 levels showed an average increasing pattern over time preceding the endpoint. In endpoint-free patients, KIM-1 levels were lower and slightly decreased during followup (Figure 2b). Independently of baseline levels, higher KIM-1 levels at any time during follow-up were associated with the endpoint (per 20% increase of KIM-1 levels: 1.08 [1.04–1.11], P < 0.001). Similarly, larger KIM-1 slope predicted the endpoint (1.64 [1.38–2.05], P < 0.001). These risk estimates remained significant after multivari-

able adjustments (Table 3). Table S3 shows similar results for HF hospitalizations, except for KIM-1 levels that lost significance after adjusting for cardiac markers.

Table 3 | Associations between tubular markers, urinary NAG and KIM-1, and the composite endpoint

	Urinary NAG		Urinary KIM-1	
	HR (95% CI)	***P* value**	**HR (95% CI)**	***P* value**
Baseline levels[a]				
Model A	1.07 (1.05–1.09)	<0.001	1.02 (1.00–1.04)	0.06
Model B	1.06 (1.03–1.09)	<0.001	1.01 (0.99–1.03)	0.26
Model C	1.03 (1.00–1.06)	0.050	0.99 (0.97–1.01)	0.44
Temporal evolution[b]				
Repeatedly measured levels				
Model 1	1.06 (1.01–1.11)	0.018	1.08 (1.04–1.11)	<0.001
Model 2	1.07 (1.03–1.12)	<0.001	1.06 (1.03–1.10)	<0.001
Model 3	1.05 (1.00–1.10)	0.048	1.04 (1.01–1.07)	0.016
Model 4	1.13 (1.09–1.17)	<0.001	1.06 (1.03–1.09)	<0.001
Annual slope				
Model 1	1.26 (1.11–1.44)	0.004	1.64 (1.38–2.05)	<0.001
Model 2	1.50 (1.18–2.00)	0.002	1.78 (1.41–2.39)	<0.001
Model 3	0.81 (0.65–1.41)	0.16	1.52 (1.25–1.98)	<0.001
Model 4	1.10 (1.02–1.20)	0.009	1.12 (1.04–1.20)	0.002
AUCm				
Model 1	1.02 (0.99–1.05)	0.11	1.01 (0.99–1.02)	0.23
Model 2	1.04 (1.01–1.07)	0.01	1.01 (0.99–1.03)	0.10
Model 3	1.01 (0.98–1.05)	0.33	1.01 (0.99–1.02)	0.38

AUCm, area under the curve of marker's trajectory; CI, confidence interval; HR, hazard ratio; KIM-1, kidney injury molecule-1; LME, linear mixed effects; NAG, N-acetyl-beta-D-glucosaminidase.

[a]HRs and 95% CIs are given per 20% increase of urinary NAG and KIM-1. Model A is unadjusted. Model B is adjusted for age, sex, diabetes, atrial fibrillation, baseline New York Heart Association class, diuretics, systolic blood pressure, and estimated glomerular filtration rate. Model C is adjusted for baseline NT-proBNP and hs-cTnT.

[b]HRs and 95% CIs are given per 20% increase of the level, slope, and AUCm of urinary NAG and KIM-1. Model 1 is Cox model–adjusted for marker's baseline levels and LME model–adjusted for sampling time. Model 2 is Cox and LME model–adjusted for age, sex, diabetes, atrial fibrillation, baseline New York Heart Association class, diuretics, systolic blood pressure, estimated glomerular filtration rate, and sampling time (LME). Model 3 is Cox and LME model–adjusted for baseline NT-proBNP and hs-cTnT and sampling time (LME). Model 4 is time-dependent Cox–adjusted for total daily equivalent doses of carvedilol, enalapril, furosemide, and spironolactone during follow-up.

Plasma and urinary NGAL. Although baseline plasma NGAL levels were higher in patients who reached the endpoint, this difference declined during follow-up (Supplementary Figure S1A). The evolution of urinary NGAL levels of patients who reached the endpoint and those who did not substantially overlapped during follow-up (Supplementary Figure S1B). No clear associations were found between NGAL and primary and secondary endpoints during follow-up (Supplementary Tables S4 and S5).

Prospective accuracy

Supplementary Table S6 shows the time-dependent area under the receiver operating curve (AUC) for the different renal markers for the composite endpoint. After the 1-year collection time period, markers showed reasonably good discriminatory power both for the 6- and 12-month risk window, with slightly better accuracy for the 6-month window. The highest accuracy was found for clinical models using levels of CysC, NAG, and KIM-1 (6-month AUCs: 0.80, 0.81, and 0.80, respectively).

Patient-specific dynamic prediction

Figure S2 shows the temporal patterns of eGFR and NAG in several individual patients from our cohort, together with their corresponding individual survival probabilities as estimated by the joint model. The figure shows that each time an additional measurement is performed in the patient, the individual survival probability is updated. Specifically, rising marker levels and worsening prognosis can be seen in the example patients who ultimately reached the composite endpoint versus stable or decreasing marker levels and more favorable prognosis in the example patients who stayed event-free.

DISCUSSION

We have shown that in patients with CHF both glomerular function (as assessed by repeatedly measured creatinine, eGFR, and CysC), and tubular function (as assessed by repeatedly measured urinary NAG and KIM-1) deteriorate over time preceding clinical outcome. Importantly, patient-specific trajectories of all glomerular markers dynamically predicted the event, and CysC was the strongest predictor. Similarly, patient-specific trajectories of urinary NAG and KIM-1 indicated progression of tubular damage in patients who later suffered adverse events. No clear associations were found between repeatedly measured plasma or urinary NGAL and the event.

Therefore, the current study does not justify its use for clinical prediction in patients with CHF.

Our findings confirm that renal function is an indivisible component of HF, and that it is clinically relevant for the monitoring of stable patients with CHF. Importantly, our results show that temporal changes in renal function remain predictive for clinical outcome despite controlling for NYHA class, cardiac markers and other clinical features, which suggests that renal dysfunction may drive adverse clinical outcomes independently of cardiac dysfunction. In addition, the results demonstrate the predictive value not only of GFR levels (single value or cumulative effects), but also of GFR slope. These findings are supported by other studies.[4,10] However, unlike previous studies, our study underscores that GFR evolution should be assessed as a function of time. In other words, information on early and late GFR changes,[12] as well as the time interval during which GFR was measured, should be taken into consideration. This recommendation is also supported by recent results from Damman et al., who found that when eGFR is assessed as a function of time, any decrease in eGFR will result in increased event rates. In previous studies, deltas in creatinine or eGFR between any 2 sampling moments were mostly used, which may have led to bias as a consequence of differences in the time periods (before the event) in which sampling was performed. In our study, the observations were made using 2 glomerular markers, creatinine and CysC, which were assessed at fixed time intervals; using more than twice as many repeated measurements as previous studies did. Notably, CysC showed the strongest association with adverse events. Considering that generation of creatinine changes when muscle wasting occurs with progression of cardiac disease, this can be of particular interest when renal function is repeatedly assessed in the same individual with CHF. Nonetheless, this issue requires further exploration.

In the setting of tubular injury, we found not only that patients with CHF experience tubular damage, but also that the damage progresses over time (months) preceding a clinical event. This extends previous findings by demonstrating that tubular markers, which were previously shown to capture acute kidney injury,[13] are also clinically relevant in chronic tubular damage in patients with CHF when followed during a prolonged time period.[11] To our best knowledge, our study is the first to simultaneously follow glomerular and tubular markers and to show that glomerular dysfunction and tubular injury, in most cases, do not progress over time in parallel. This implies that, although the failing heart affects both renal compartments, the degree of damage in these compartments is usually not temporally coupled. There-

fore, they should be viewed as different renal entities in CHF. In addition, when we examined NAG and KIM-1, we found that NAG levels will rise first, followed by a rise in KIM-1. This suggests that, although both markers are labeled as "tubular damage markers," they reflect different biological aspects of tubular injury, and their values depend on the moment in time prior to the event at which they are assessed. These findings are in line with their behavior as previously found. Increased urinary excretion of NAG has been found to occur with abnormal increases in protein traffic across the proximal tubules as a consequence of a damaged glomerular barrier.[14] On the other hand, KIM-1 gene expression has been found to be upregulated in a dose-dependent manner in response to direct tubular injury.[15] KIM-1 also correlated strongest with tubular damage as determined by kidney biopsies. It outperformed serum creatinine, blood urea nitrogen (BUN), and urinary NAG.[16,17] Thus, it appears that NAG is a marker of tubular dysfunction that shows an early initial rise, while KIM-1 can serve as a quantitative marker of tubular damage, if modeled in a time-dependent manner. Importantly, both tubular markers are relevant for clinical outcomes.

The unique advantages of our study include frequent repeated measurements at pre-specified time intervals (i.e., sampling was not left at the discretion of the treating physicians) during longer-term follow-up. This allowed us to provide an unbiased assessment of a patient's risk by using the complete temporal biomarker trajectory as assessed over the entire follow-up period. Based on this underlying trajectory, biomarker levels are used to estimate the risk of future adverse events.[18] Herewith, a window of opportunity may be gained to modify the treatment before a future event occurs. Joint modeling (JM) of patient-specific marker trajectories and survival analysis enables us to perform individualized risk predictions based on individual biomarker values. Subsequently, predictions are dynamically updated to provide real-time risk assessment whenever extra information is collected.[19] Such dynamic risk profiling can enable physicians to better detect disease progression and to make wellinformed individualized treatment decisions. Applicability of JM in daily practice is user-friendly, and an app is already available into which a patient's data (baseline and follow-up) can be uploaded (for details please see Figure S3).[20]

Study limitations

Firstly, our cohort consisted mainly of patients with HF and reduced ejection fraction. The low number of patients with HF and a preserved ejection fraction can most likely be attributed to the fact that in the Netherlands, most such patients

are treated by the general practitioner or in secondary referral centers, while the current study was performed in 2 centers that were both tertiary referral centers. Potential inclusion bias is not a likely reason for the low HF and preserved ejection fraction rate, because all consecutive patients were screened in both participating centers. Secondly, enrolled CHF patients were in a better health condition than previously reported CHF populations. Yet, we were able to demonstrate, even in this "less sick" CHF population that evolutions of glomerular and tubular dysfunction predict clinical outcome. Thus, it is possible that these markers could perform even better in more sick CHF patients. Thirdly, although we adjusted for several confounders, residual confounding may be present. However, we corrected all urinary markers for concentration or dilution of urine caused by diuretics during follow-up. Furthermore, treating physicians were blinded to biomarker data to exclude bias by treatment effect. Finally, although our findings underscore the importance of regular monitoring of both glomerular and tubular function in CHF, routine evaluation of kidneys should always be seen in the light of the patient's clinical status.

Altogether, our findings demonstrate that glomerular function (as assessed by creatinine, eGFR, and CysC), and tubular function (as assessed by urinary NAG and KIM-1) deteriorate, but not simultaneously, during clinically silent progression of CHF over time preceding adverse events. Patient-specific temporal evolutions of these repeatedly measured renal markers dynamically predict clinical outcome in CHF patients, and are useful for individual risk profiling.

MATERIALS AND METHODS

The Bio-SHiFT study is a prospective, observational cohort study of stable patients with CHF, conducted at Erasmus Medical Center (Rotterdam, Netherlands) and Noordwest Ziekenhuisgroep (Alkmaar, Netherlands). Patients were recruited during their regular visits to the cardiology outpatient clinics of these hospitals. For this purpose, consecutive patients were screened according to the inclusion and exclusion criteria specified in Supplementary Figure S1, and eligible patients were asked for informed consent. The main inclusion criteria were age ≥18 years, capability of understanding and signing informed consent, and diagnosis of CHF ≥3 months ago according to European Society of Cardiology guidelines (Supplementary Figure S4).[21,22] Patients were ambulatory and stable (i.e., they had not been hospitalized for HF in the past 3 months). The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered

with ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients who participated in the study. This investigation comprised 263 stable patients with CHF enrolled during the first inclusion period (October 2011 until June 2013).

Baseline assessment

All patients were evaluated by research physicians, who collected information on HF-related symptoms, NYHA class, and performed a physical examination, including blood pressure, heart rate, and body mass index. Information on HF etiology, left ventricular ejection fraction, cardiovascular risk factors, medical history, and medical treatment was retrieved primarily from hospital records and was checked if ambiguities were present. History of cardiovascular and other comorbidities was defined as a clinical diagnosis of these conditions. Non-fasting blood and urine samples were collected, as described below.

Follow-up and study endpoints

During the study, all patients were routinely followed at the outpatient clinic by treating physicians who were blinded to biomarker sampling and results. Study follow-up visits were predefined and scheduled every 3 months (±1 month was allowed), with a maximum of 10 study follow-up visits. At each study follow-up visit, a short medical evaluation was performed and samples were collected. All medication changes and occurrence of adverse cardiovascular events since the previous visit were recorded in electronic case report forms. During follow-up, hospitalizations for HF, myocardial infarction, percutaneous coronary intervention, coronary artery bypass surgery, arrhythmias, cerebrovascular accident, cardiac transplantation, LVAD implantation, and mortality were recorded in the electronic case report forms, and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker sampling and results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

The primary endpoint comprised the composite of cardiac death, cardiac transplantation, LVAD implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. Secondary endpoints included individual components of the primary endpoint, and also myocardial infarction, percutaneous coronary intervention, coronary artery bypass surgery, cerebrovascular accident, and all-cause mortality. Cardiac death was defined as death from myocardial in-

farction or other ischemic heart disease (ICD-10: I20-I25), death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96) or unwitnessed or ill-described death (R98, R99). Hospitalization for acute or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with 2 of the following: BNP or NT-proBNP > 3x ULN, signs of worsening HF, such as pulmonary rales, raised jugular venous pressure or peripheral edema, increased dose or i.v. administration of diuretics, or administration of positive inotropic agents.[21]

Blood and urine analysis

Blood and urine samples were collected at baseline and at each study follow-up visit and were processed and stored at a temperature of –80°C within 2 hours after collection. The biomarker measurements performed for this study did not lead to drug adjustments and all patients received usual care. Batch analysis of plasma and urine samples was performed at HaemoScan BV (Groningen, Netherlands) using methods described in the supplementary text. All urinary biomarkers were normalized to urinary creatinine concentrations to correct for concentration or dilution of urine.

GFR was determined by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation that has been validated in HF patients.[23] Patients were categorized using National Kidney Foundation–Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines.[24]

Statistical analysis

Biomarkers measured at baseline. The association between baseline marker levels and the study endpoint was examined by Cox regression analysis. If skewed, ^{2}log-tranformation of continuous variables was used for further analyses. Analyses were first performed univariably, then statistical adjustments were performed by using 2 models: (i) model with biomarker of interest plus clinical variables age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, and eGFR (for tubular markers); and (ii) model with biomarker of interest plus biomarkers of myocardial stretch and damage, NT-proBNP, and hs-cTnT. Data on all variables were complete, except for systolic blood pressure, which was missing in <5% of patients and for which imputations were applied using patients' clinical and outcome data. The proportional hazards (PH) assumption was evaluated by plotting transformed Kaplan-Meier estimates, and by evaluating scaled Schoenfeld residuals.

Repeatedly measured biomarkers. We applied a joint modeling (JM) of linear mixed-effects (LME) models to assess the true underlying trajectory of a repeatedly measured marker, and a Cox survival analysis to analyze the association of this trajectory with the study endpoint. For both the fixed- and random-effects parts of LME, nonlinear evolutions were tested using restricted cubic splines. If the model was not significantly improved, a linear evolution was retained. All markers were adjusted for the sampling time during follow-up. Additional statistical adjustments were as follows: (i) the repeatedly measured marker was adjusted for its baseline level (Cox model) to examine incremental value of repeated over baseline measurements; (ii) Cox and LME models were adjusted for the clinical variables age, sex, diabetes, atrial fibrillation, NYHA class, diuretics, systolic blood pressure, and eGFR (for tubular markers) to examine incremental value of the renal markers over the patients' clinical characteristics; (iii) Cox and LME models were adjusted for biomarkers of myocardial stretch and damage (NT-proBNP and hs-cTnT) to examine the incremental value of the renal markers over these commonly used cardiac markers. Results are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) per 20% change in biomarkers levels.

To investigate the independent predictive value of these renal markers on the study endpoints, all individual temporal biomarker patterns derived from the joint models were extracted and subsequently entered simultaneously with HF medication doses (repeatedly assessed during follow-up) into a time-dependent Cox analysis.

Parameterization of marker's trajectory. The above-described analyses estimate the instantaneous risk based on repeatedly measured marker levels. However, in the context of repeated measurements, we also estimated the following aspects:[25,26] (i) the time-dependent slope (or rate of change) of the marker's trajectory, indicating whether and by how much the levels are increasing or decreasing at any point in time, which corresponds to the first derivative of the marker's trajectory; and (ii) the AUCm, indicating the cumulative effect of all the values the marker has taken in the past (Figure 3). The results are presented as HRs [95% CI] per 20% change in the annual slope (delta of the marker's levels/year) and the AUCm.

Prospective accuracy. We determined the longitudinal marker's predictive accuracy (i.e., the ability of a marker to discriminate between a patient who experiences the event within a given time window after the last measurement and the patient who does not experience the event within that same time window) using the time-dependent AUC methodology.[19] For this purpose, we chose the first year as the

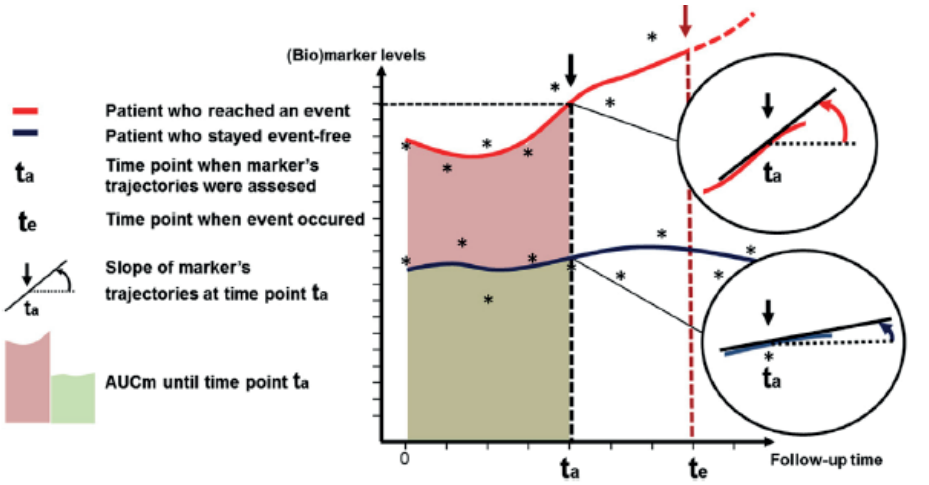


Figure 3 | Dynamic risk prediction model using repeated marker measurements. An illustration of the underlying trajectory of a repeatedly assessed biomarker in a patient who ultimately experiences the event (solid red line) and in an event-free patient (solid blue line). Marker's levels (asterisks) are displayed on the y-axis and follow-up time on the x-axis. The figure shows different types of parameterization that can be examined: marker's levels at any point in time (ta), slope of the marker's trajectory at any point in time (ta), and the area under the curve of marker's trajectory (AUCm) up to the same point in time (ta). te, time when the event occurred.

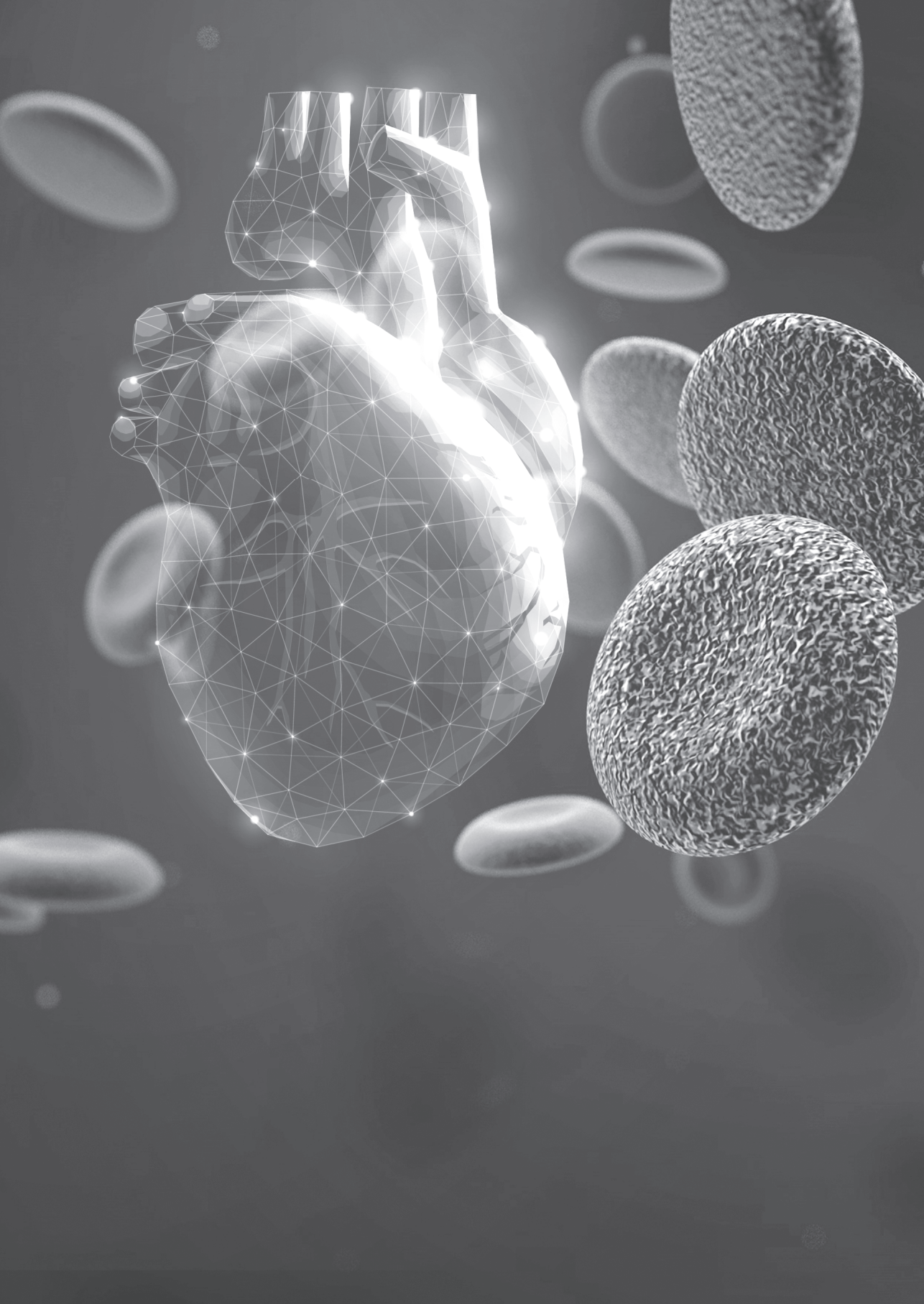
collection time period, and we assessed 2 risk time-windows: 6 and 12 months after the collection time.

All analyses were performed with R package JMbayes statistical software.[18,26] All tests were 2-tailed, and P values < 0.05 were considered statistically significant.

REFERENCES

1. Burchfield JS, Xie M, Hill JA. Pathological ventricular remodeling: mechanisms: part 1 of 2. Circulation. 2013;128:388–400.
2. Eapen ZJ, Liang L, Fonarow GC, et al. Validated, electronic health record deployable prediction models for assessing patient risk of 30-day rehospitalization and mortality in older heart failure patients. JACC Heart Fail. 2013;1:245–251.
3. Ponikowski P, Voors AA, Anker SD, et al. 2016 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure: the task force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J. 2016;37:2129–2200.
4. Damman K, Valente MA, Voors AA, et al. Renal impairment, worsening renal function, and outcome in patients with heart failure: an updated meta-analysis. Eur Heart J. 2014;35:455–469.
5. Dries DL, Exner DV, Domanski MJ, et al. The prognostic implications of renal insufficiency in asymptomatic and symptomatic patients with left ventricular systolic dysfunction. J Am Coll Cardiol. 2000;35:681–689.
6. Hillege HL, Girbes AR, de Kam PJ, et al. Renal function, neurohormonal activation, and survival in patients with chronic heart failure. Circulation. 2000;102:203–210.
7. Goldfarb M, Abassi Z, Rosen S, et al. Compensated heart failure predisposes to outer medullary tubular injury: studies in rats. Kidney Int. 2001;60:607–613.
8. Tsuruya K, Eriguchi M. Cardiorenal syndrome in chronic kidney disease. Curr Opin Nephrol Hypertens. 2015;24:154–162.
9. Damman K, Voors AA, Navis G, et al. Current and novel renal biomarkers in heart failure. Heart Fail Rev. 2012;17:241–250.
10. Damman K, Masson S, Hillege HL, et al. Tubular damage and worsening renal function in chronic heart failure. JACC Heart Fail. 2013;1:417–424.
11. Damman K, Masson S, Hillege HL, et al. Clinical outcome of renal tubular damage in chronic heart failure. Eur Heart J. 2011;32:2705–2712.
12. Levey AS, Inker LA, Matsushita K, et al. GFR decline as an end point for clinical trials in CKD: a scientific workshop sponsored by the National Kidney Foundation and the US Food and Drug Administration. Am J Kidney Dis. 2014;64:821–835.
13. Siew ED, Ware LB, Ikizler TA. Biological markers of acute kidney injury. J Am Soc Nephrol. 2011;22:810–820.
14. Skalova S. The diagnostic role of urinary N-acetyl-beta-Dglucosaminidase (NAG) activity in the detection of renal tubular impairment. Acta Medica (Hradec Kralove). 2005;48:75–80.
15. Chiusolo A, Defazio R, Zanetti E, et al. Kidney injury molecule-1 expression in rat proximal tubule after treatment with segment-specific nephrotoxicants: a tool for early screening of potential kidney toxicity. Toxicol Pathol. 2010;38:338–345.
16. Vaidya VS, Ozer JS, Dieterle F, et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. Nat Biotechnol. 2010;28:478–485.
17. Tonomura Y, Tsuchiya N, Torii M, et al. Evaluation of the usefulness of urinary biomarkers for nephrotoxicity in rats. Toxicology. 2010;273: 53–59.

18. Rizopoulos D. Joint Models for Longitudinal and Time-to-Event Data: With Applications in R. Boca Raton: Chapman & Hall/CRC; 2012.
19. Rizopoulos D.Dynamic predictions and prospective accuracy in jointmodels for longitudinal and time-to-event data. Biometrics. 2011;67:819–829.
20. https://github.com/drizopoulos/JMbayes.
21. McMurray JJ, Adamopoulos S, Anker SD, et al. ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J. 2012;33:1787–1847.
22. Paulus WJ, Tschope C, Sanderson JE, et al. How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. Eur Heart J. 2007;28:2539–2550.
23. McAlister FA, Ezekowitz J, Tarantini L, et al. Renal dysfunction in patients with heart failure with preserved versus reduced ejection fraction impact of the new chronic kidney disease-epidemiology collaboration group formula. Circ-Heart Fail. 2012;5:309–314.
24. National Kidney F. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002;39:S1–S266.
25. Rizopoulos D, Takkenberg JJ. Tools & techniques–statistics: dealing with time-varying covariates in survival analysis–joint models versus Cox models. EuroIntervention. 2014;10:285–288.
26. Rizopoulos D. The R package JMbayes for fitting joint models for longitudinal and time-to-event data using MCMC. Journal of Statistical Software. 2016;72:46.



Summary and conclusions
Clinical perspectives and future directions
Dutch summary (Nederlandse samenvatting)
List of publications
Author information
Acknowledgements (Dankwoord)
PhD portfolio

SUMMARY AND CONCLUSIONS

In order to fully utilize the opportunities of a personalized medicine model in coronary artery disease (CAD) and heart failure (HF), our understanding of the (adverse) disease course in individual patients must be improved. The cohesive research projects that compose this thesis aimed to investigate if, and to what extent, serum biomarkers and intracoronary imaging may contribute to that goal. We now summarize our main findings.

Part I - Blood biomarkers and novel imaging techniques in coronary artery disease

The first part of this thesis focused on the role of blood biomarkers and intracoronary imaging characteristics in CAD patients. For this purpose, investigations were performed in *the European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis - IntraVascular UltraSound* (ATHEROREMO-IVUS) study and *the Integrated Biomarker and Imaging Study 3* (IBIS-3).

ATHEROREMO-IVUS was designed to evaluate the association between novel circulating biomarkers, coronary plaque characteristics as assessed by radiofrequency- (RF-)IVUS and near-infrared spectroscopy system (NIRS), and the occurrence of major adverse cardiac events (MACE), defined as the composite of all-cause mortality, nonfatal acute coronary syndrome (ACS) or unplanned coronary revascularization. IVUS and NIRS were performed in a 40 mm long non-culprit coronary artery segment in patients referred for coronary angiography (CAG) or percutaneous coronary intervention (PCI) for stable angina pectoris (SAP) or ACS. ATHEROREMO-IVUS enrolled 768 patients; NIRS was performed in a subset of 203.

IBIS-3 study is a prospective, observational cohort, in which the effect of high intensity statin therapy was evaluated through repeated IVUS (N=164) and NIRS (N=103) measurements of a non-stenotic coronary artery segment, and repeated blood sampling, both performed at baseline and 1-year follow-up.

In **Chapter 2** we described the association between atherosclerotic burden derived from all three coronary arteries, as assessed by the CAG-based anatomic Syntax score (SXscore), and atherosclerotic burden as assessed by RF-IVUS and NIRS in a single non-culprit segment. For the selection of the optimal revascularization strategy - and thus for optimal risk reduction on the longer term - adequate quantification of the complexity of CAD is required. Several scores have been developed for this purpose, and the SXscore is a well-established example. This score takes into account the number of significant lesions and their location, but also the

complexity of each lesion independently. We investigated whether the SXscore is correlated with information on the extent and phenotype of coronary atherosclerosis as provided by the intracoronary imaging modalities RF-IVUS and NIRS. We found that a higher SXscore was associated with higher RF-IVUS- and NIRS-derived atherosclerotic burden. While the SXscore lacks detail with respect to coronary artery wall pathology, RF-IVUS and NIRS have been shown to provide information on plaque morphology in the imaged coronary segment. Thus, our results show a direct association between the angiographic atheroma burden of all three vessels and intravascular coronary wall evaluation of a non-culprit segment. These findings underline the value of RF-IVUS and NIRS derived measures for risk assessment of CAD patients.

Although intracoronary imaging techniques provide valuable tools for risk assessment, blood biomarkers may carry potential to detect vulnerable plaques in an early stage and in a non-invasive manner. **Chapters 3-5** describe the associations between circulating biomarkers of inflammation, renal function and lipid metabolism, with coronary lipid core burden index (LCBI) assessed by NIRS imaging, as well as the association of these biomarkers with the occurrence of MACE during (a median of 4.7 year) follow-up in ATHEROREMO-IVUS.

In **Chapter 3**, we studied 26 inflammatory (acute phase proteins, cytokines and chemokines) and renal biomarkers, using a validated multiplex assay (Myriad RBM). After adjustment for clinical characteristics, TNF-α appeared the only biomarker that tended to be associated with higher LCBI, although this association did not persist after correction for multiple testing. Thus, the multiplex panel that we used did not render a useful blood biomarker of high LCBI. Moreover, from this multiplex panel only IL-8 was independently associated with MACE.

In **Chapter 4** we studied the prognostic value of ten previously identified high-risk molecular lipid species and three ceramide ratios. We found that plasma Cer(d18:1/16:0) was associated with MACE, independent of statin use and serum LDL-C level. Furthermore, after multivariable adjustment, concentrations of Cer(d18:1/16:0), Cer(d18:1/20:0), Cer(d18:1/24:1), and all investigated ceramide ratios were associated with the composite of all-cause mortality or nonfatal ACS, our secondary endpoint. Our results support the hypothesis that plasma concentrations of ceramides and their ratios are predictive of long-term cardiovascular outcome in patients with established CAD.

The proprotein convertase subtilisin/kexin type 9 (PCSK9) enzyme induces LDL-receptor degradation in the liver, resulting in an increase in circulating serum LDL-C

levels that promote atherosclerosis. In **Chapter 5**, we demonstrated that higher serum levels of PCSK9 were associated with higher NIRS-derived LCBI, as well as with a high MACE incidence. Our finding indirectly supports previous observations, showing that serum PCSK9 levels directly contribute to inflammation of the atherosclerotic plaque, and may thus reflect the vulnerability of the entire coronary tree. Herewith, our findings imply that PCSK9 inhibition may exert its beneficial therapeutic effects in CAD patients, not only by means of its LDL-C lowering, but also by its anti-inflammatory properties.

In **Chapter 6** we focused on inflammation, and studied changes in serum CRP levels, serum cholesterol levels and plaque characteristics in relation to rosuvastatin treatment (the IBIS-3 study). In patients with established CAD, we found clinically relevant reductions in CRP levels after 1 year intensive (40 mg daily) rosuvastatin therapy. These changes were observed in SAP as well as in ACS patients, but most prominent in ACS. Furthermore, in the ACS patients that we studied, CRP changes were correlated with changes in IVUS-derived plaque characteristics. We found no such correlations in SAP patients. CRP changes were uncorrelated with the also observed reductions in LDL-C. Hence, our study supports the role of inflammation in CAD progression, but also emphasizes that the relation between LDL-C reduction, inhibition of (vascular) inflammation, changes in extent and composition of coronary plaques, and coronary event reduction is not straightforward.

Finally, in **Chapter 7** we move to clinical practice in the context of CAD and describe the implementation of a new electrocardiogram (ECG) electronic transmission (e-transmission) system in the Rotterdam region. In pre-hospital settings handled by paramedics, identification of patients with myocardial infarction (MI) remains challenging when automated ECG interpretation is inconclusive. In December 2013 we supplemented the automated ECG-devices on the ambulances with a modem, which enabled e-transmission of the ECGs for expert consultation. Thus we modified the diagnostic protocol for patients with acute ischemic chest pain by incorporating this technical option. The study cohort comprised 1421 patients. During a 1-year evaluation period, applying the modified pre-hospital triage protocol, 115 acute MI patients with an initially inconclusive ECG received primary PCI within 90 min, whereas another 20 received PCI within 90-120 min. Since our study was not a randomized trial or before-after study, we cannot conclude with entire certainty that the observed early treatment was the direct consequence of a change in patient flow that was induced by the new triage protocol. Still, based on the obtained results, we have decided to continue the use of the modified protocol.

Part II - Blood biomarkers in heart failure

The second part of the thesis "*Blood biomarkers in heart failure*" focused on the additional value of blood biomarkers in patients with HF. For this purpose, investigations were performed in the *Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis* (Bio-SHiFT) study. The Bio-SHiFT study is a prospective, observational cohort of 263 clinically stable patients with chronic heart failure (CHF) conducted in the Erasmus MC, Rotterdam, and Noordwest Ziekenhuisgroep, Alkmaar. Patients were recruited during their regular outpatient visits and were in clinically stable condition. At baseline, and subsequently every 3 months (until 30 months), blood and urine samples were collected and clinical status (including NYHA class) was assessed. This repeated-measures design allowed us to explore in detail the temporal trajectories of a wide range of biomarkers during the course of CHF.

In **Chapter 8** we describe the associations between repeatedly measured NT-proBNP, high-sensitivity troponin T (Hs-TnT) and CRP with NYHA class, as well as with adverse clinical outcomes during a median follow-up of 1 year. Temporal patterns of NT-proBNP and Hs-TnT were positively associated with repeatedly assessed NYHA class. Moreover, temporal patterns of NT-proBNP, CRP and NYHA class were independently associated with the composite endpoint of cardiovascular mortality, heart failure hospitalization, cardiac transplantation or left ventricular assist device implantation. Serial assessments of NT-proBNP and CRP both added value to clinical characteristics in terms of discriminative ability. Serially measured NT-proBNP in combination with NYHA class seemed best suited for monitoring CHF outpatients. Altogether, our findings underscore the incremental value of biomarkers to NYHA class for monitoring stable CHF outpatients.

In **Chapter 9** we extended the follow-up to a median of 2.2 years to further explore the associations between repeatedly measured NT-proBNP, Hs-TnT and CRP with adverse clinical outcomes. We confirmed the findings that we described in Chapter 8. Using a joint modeling approach, we found that not only the temporal evolution of biomarker levels, but also the instantaneous rate of change in level (i.e. the instantaneous slope of the temporal pattern) of NT-proBNP and CRP was associated with adverse clinical outcomes, as was the area under the curve of the trajectory of NT-proBNP. Our findings suggest that individual patterns of change of biomarkers, as well as combinations of multiple biomarkers, may be clinically relevant as they may further refine estimation of a patient's prognosis.

MicroRNAs (miRs) are upcoming novel biomarkers that seem promising for early diagnosis and treatment of HF. MiRs are non-coding, ~22 nucleotide long RNA sequences, which target messenger RNAs for cleavage or translational repression and thereby influence a great variety of biological processes. In **Chapter 10** we describe the association of repeated measurements of 7 MiRs that were previously linked to HF (miR-1254, miR-22-3p, miR-423-5p, miR-486-5p and miR-320a) or have been shown to be cardiac-enriched (miR-345-5p, miR-378a-3p), with adverse clinical events in CHF patients. We found that the temporal pattern of circulating miR-22-3p was associated with adverse outcome. The association was independent of clinical characteristics and temporal NT-proBNP, HsTNT and CRP patterns. The instantaneous rate of change in miR-22-3p level was also inversely associated with adverse outcome. Moreover, we found inverse associations of temporal patterns of miR-22-3p with temporal patterns of HsTNT and CRP. Thus, the use of individual patterns of change of circulating miR-22-3p may potentially be useful for CHF prognostication.

Although it is known that chronic kidney disease is frequently present in patients with CHF, the detailed temporal evolutions of glomerular and tubular markers in CHF have never been described. In **Chapter 11** we demonstrated that in patients with CHF, glomerular function (as assessed by creatinine, estimated glomerular filtration rate (eGFR) and cystatin C (CysC)), and tubular function (as assessed by urinary N-acetyl-beta-D-glucosaminidase (NAG) and kidney-injury-molecule (KIM-1)) deteriorate during clinically silent progression of CHF. Moreover, patient-specific temporal evolutions of these repeatedly measured renal markers predict adverse clinical outcomes in CHF patients, independently of the patients clinical characteristics, pharmacological treatment, cardiac natriuretic peptides and troponins, and for tubular markers also independently of eGFR. To our best knowledge, this study is the first to simultaneously and repeatedly asses glomerular and tubular function over time during long-term follow-up, and to show that both renal compartments chronically deteriorate during progression of HF. These renal biomarker levels and their rates of change may be useful for dynamic risk profiling, which can support physicians in improved detection of disease progression and in making personalized treatment decisions.

MAIN CONCLUSIONS

The main conclusions of the thesis can be summarized as follows:

Part 1, coronary artery disease

- Higher SXscore is associated with higher atherosclerotic burden as assessed by NIRS and RF-IVUS in a single non-stenotic coronary artery segment in patients with CAD.
- A multiplex panel of 26 inflammatory biomarkers (acute phase proteins, cytokines, and chemokines) and renal markers did not render a useful blood biomarker of high NIRS-derived LCBI. Conversely, circulating serum PCSK9 levels were positively associated with LCBI. This association was independent of established cardiac risk factors, statin use and serum LDL-C.
- Plasma IL-8, plasma Cer(d18:1/16:0) and serum PCSK9 levels were independently associated with adverse cardiovascular outcomes during a median follow-up period of 4.7 years in patients with CAD.
- After 1 year intensive rosuvastatin therapy clinically relevant reductions in CRP levels were observed in a series of patients with established CAD. The observed CRP changes were correlated with changes in IVUS-derived plaque characteristics in ACS patients, but not in SAP. CRP changes were uncorrelated with changes in LDL-C levels.

Part II, heart failure:

- Serially assessments NT-proBNP and Hs-TnT were positively associated with serially assessed NYHA class in patients with stable CHF. Repeatedly measured NT-proBNP and CRP both add individually to serial NYHA-class assessments for monitoring CHF patients in terms of discriminative ability compared to a model with only serial NYHA class measurements.
- The dynamic, temporal patterns of serially measured NT-proBNP and CRP are strong and independent predictors of adverse clinical events in patients with stable CHF. Not only the evolution of biomarkers level, but also their instantaneous rate of change in NT-proBNP and CRP levels as well as the area under the curve of the trajectory of NT-proBNP, were associated with adverse outcome.
- The temporal pattern of circulating miR-22-3p is a strong and independent predictor of prognosis in CHF patients.
- Repeatedly assessed glomerular function (creatinine, eGFR and CysC), and tubular function (NAG and KIM-1) independently predicts adverse clinical outcomes in CHF patients. Both renal compartments chronically deteriorate, but not in parallel, during clinically silent progression of CHF.

CLINICAL PERSPECTIVES AND FUTURE DIRECTIONS

The view on pathophysiology of coronary artery disease (CAD) has undergone an evolution during the past decades. It has shifted from a chronic, steady and progressive cholesterol storage disease towards a multifactorial, dynamic disease. CAD is complex and heterogeneous in nature, as it is caused by multiple genetic, environmental and behavioural factors. Therefore CAD patients should be treated using an individual approach to improve their quality of life, reduce mortality- and hospitalisation rates but also to reduce healthcare costs. In order to fully utilize the opportunities of a personalized medicine model, our understanding of the (adverse) disease course in individual patients must be improved. Further improvements in our understanding of coronary pathophysiology and the prediction of an adverse disease course in individual patients could be established by continuing investigations on the additional value of blood biomarkers and intracoronary imaging in CAD patients.

In this thesis, in patients with established CAD, the invasive coronary imaging modalities RF-IVUS and NIRS are shown to provide valuable information on plaque morphology in the imaged coronary segment and to be promising tools for risk prediction. Moreover, several circulating blood biomarkers are shown to be associated with adverse cardiovascular outcomes during long term follow-up. The major advantage of blood biomarkers is that they can be measured in a non-invasive manner and repeatedly over time. Further studies on the value of repeated biomarker measurements are warranted. In the future, studies combining clinical characteristics with repeated biomarker measurements and with repeated intracoronary imaging in a prognostic model may even further improve individual risk prediction. Such a prognostic tool may better identify 'high risk' patients and may thus facilitate clinical decision making.

In this thesis we show that in patients with stable CHF, temporal patterns of several circulating biomarkers have incremental value for prognostication. Herewith these temporal patterns carry potential to accurately detect disease dynamics, which could be useful for tailored adjustment of treatment. This thesis provides a basis for future, larger studies to confirm the value of temporal patterns of these blood biomarkers for prognostication, as well as for studies on tailored, biomarker-guided adjustment of treatment strategy.

Although the findings of this thesis are promising, it is essential that they are confirmed in large, prospective clinical studies. Thus, although much progress has been made, substantial work remains to be done in this field of personalized medicine.

NEDERLANDSE SAMENVATTING

Een ongunstig ziektebeloop komt nog vaak voor in patiënten met coronaire hartziekte (CAD) en patiënten met hartfalen (HF). Om de risicovoorspelling in deze patiëntengroepen verder te verbeteren, zijn nieuwe methoden nodig die uitgaan van zogenaamde 'gepersonaliseerde geneeskunde'. De samenhangende onderzoeksprojecten in dit proefschrift zijn bedoeld om te evalueren of, en in welke mate, serum biomarkers en intracoronaire beeldvorming kunnen bijdragen aan verbetering van de risico stratificatie en behandelstrategie van deze patiënten. In dit hoofdstuk vatten we de belangrijkste resultaten van dit proefschrift samen.

Deel I Bloed biomarkers en nieuwe beeldvormende technieken bij coronaire hartziekte

In het eerste deel van dit proefschrift genaamd *"Bloed biomarkers en nieuwe beeldvormende technieken bij coronaire hartziekte"* ligt de nadruk op additionele waarde van serum biomarkers en intracoronaire plaque karakteristieken in patiënten met coronarialijden binnen de European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis - IntraVascular UltraSound (ATHEROREMO-IVUS) studie en de Integrated Biomarker and Imaging Study 3 (IBIS-3).

De ATHEROREMO-IVUS studie heeft als doel om de associatie tussen circulerende serum biomarkers en intracoronaire plaque karakteristieken bepaald met radiofrequentie(RF)-IVUS en near-infrared spectroscopy (NIRS) te bestuderen. Verder heeft deze studie als doel om de prognostische waarde van de circulerende serum biomarkers en intracoronaire plaque karakteristieken voor ongunstige cardiovasculaire uitkomsten op een relatief lange termijn (1-4 jaar) te evalueren.

De invasieve afbeelding van de kransslagaders is uitgevoerd door middel van IVUS en NIRS in een "non-culprit" (niet acuut afgesloten) coronair segment van 40 mm lang bij patiënten die verwezen waren voor coronair angiografie (CAG) of percutane coronaire interventie (PCI) vanwege stabiele angina pectoris (SAP) of acuut coronair syndroom (ACS). Er namen 768 patiënten deel aan de ATHEROREMO-IVUS studie. Intracoronaire beeldvorming middels IVUS is in 581 patiënten verricht; NIRS werd uitgevoerd in een subset van 203 patiënten.

Het IBIS-3-onderzoek is een prospectieve studie waarin het effect van statine therapie op de kransslagaders werd geëvalueerd d.m.v. herhaalde IVUS (N = 164) en NIRS (N = 103) metingen, bij aanvang van de studie en na 1 jaar follow-up.

Hoofdstuk 2 beschrijft de associatie tussen atherosclerotische karakteristieken van alle drie de kransslagaders, bepaald met de CAG-gebaseerde anatomische

Syntax-score (SXscore), en intracoronaire atherosclerotische karakteristieken, beoordeeld met RF-IVUS en NIRS, in een enkel niet-culprit coronair segment. Voor een optimale revascularisatie strategie - en dus voor een optimale risicoreductie op langere termijn - is een adequate kwantificering van de complexiteit van CAD vereist. Er zijn verschillende scores ontwikkeld en de SXscore is daar een goed voorbeeld van. SXscore houdt rekening met het aantal significante laesies en hun locatie, en ook met de complexiteit van elke laesie, onafhankelijk van elkaar. We hebben onderzocht of de SXscore verband houdt met de mate en het fenotype van coronaire atherosclerose, vastgesteld met intracoronaire beeldvorming (RF-IVUS en NIRS). We ontdekten dat een hogere SXscore geassocieerd was met een hogere mate van intracoronaire atherosclerose vastgesteld met RF-IVUS- en NIRS. Onze resultaten laten hiermee een directe associatie zien tussen de angiografisch vastgestelde atheroom belasting van alle drie de vaten en de intracoronaire atherosclerotische karakteristieken van een niet-culprit segment. Deze bevindingen onderstrepen de waarde van van RF-IVUS en NIRS voor risicobeoordeling van patiënten met stabiel CHF.

Hoewel intracoronaire beeldvormingstechnieken waardevolle instrumenten zijn voor het detecteren van hoog risico coronaire plaque, kunnen biomarkers in het bloed de potentie hebben om kwetsbare plaques in een vroeg stadium en op een niet-invasieve wijze te detecteren. Hoofdstukken 3-5 beschrijven de associaties van verschillende circulerende biomarkers met coronaire plaque karakteristieken, specifiek LCBI waarden, bepaald met NIRS beeldvorming in een non-culprit coronair segment; evenals de associatie van deze biomarkers met ongunstige klinische (cardiovasculaire) uitkomsten gedurende lange termijn follow-up (met een mediaan van 4.7 jaar) in de ATHEROREMO-IVUS studie.

Hoofdstuk 3 beschrijft de associatie tussen 26 inflammatoire (acute fase eiwitten, cytokines en chemokines) en renale biomarkers gemeten met behulp van een gevalideerde multiplex assay (Myriad RBM) en coronaire plaque karakteristieken gemeten met NIRS in patiënten met coronarialijden. Na correctie voor klinische kenmerken bleken TNF-α waarden geassocieerd te zijn met hogere LCBI waarden, echter deze associatie hield geen stand na correctie voor meervoudig testen. Kortom, het multiplex-panel dat we hier hebben gebruikt leverde geen bruikbare bloed biomarker op van hoge LCBI waarden. Bovendien was van dit multiplexpanel alleen IL-8 onafhankelijk geassocieerd met ongunstige cardiovasculaire uitkomsten.

Hoofdstuk 4 beschrijft de prognostische waarde van 10 eerder geïdentificeerde hoog risico moleculaire lipide soorten en 3 ceramide ratio's voor lange termijn car-

diovasculaire uitkomsten bij patiënten met coronarialijden. Plasma Cer (d18: 1/16: 0) bleek geassocieerd met ongunstige cardiovasculaire uitkomsten, onafhankelijk van statinegebruik en serum LDL-C-spiegels. Na multivariabele correctie, waren de concentraties van Cer (d18: 1/16: 0), Cer (d18: 1/20: 0), Cer (d18: 1/24: 1) en alle onderzochte ceramide ratio's geassocieerd met slechtere klinische uitkomsten (mortaliteit door alle oorzaken of niet-fatale ACS, ons secundaire eindpunt). Deze resultaten ondersteunen de hypothese dat plasmaconcentraties van ceramiden en hun ratio's voorspellend zijn voor lange-termijn cardiovasculaire uitkomsten bij patiënten met coronarialijden.

Het proproteïne convertase subtilisine / kexine type 9 (PCSK9) enzym induceert LDL-receptor afbraak in de lever, wat resulteert in een verhoging van de circulerende serum LDL-C-spiegels die atherosclerose bevorderen. **Hoofdstuk 5** beschrijft de associatie tussen serum PCSK9 waarden met LCBI waarden gemeten met NIRS, en met klinische uitkomsten op de lange termijn. Hoge serum PCSK9 levels waren positief en onafhankelijk geassocieerd met hogere LCBI waarden, evenals met slechtere klinische uitkomsten bij een mediane follow-up periode van 4.7 jaar na de indexprocedure bij patiënten met CAD die een CAG ondergingen omwille van ACS of SAP. Deze associatie was onafhankelijk van cardiale risicofactoren, evenals van serum LDL-C spiegels en statine gebruik. Onze bevinding ondersteunt indirect eerdere waarnemingen, die erop wezen dat serum PCSK9 waarden ontsteking van de atherosclerotische plaque op directe wijze kunnen beïnvloeden, en dus de kwetsbaarheid van de coronaire vaten kunnen weergeven. Onze bevindingen suggereren dat de gunstige effecten van PCSK9-remming bij CAD-patiënten niet alleen door LDL-C-verlaging, maar ook door ontstekingsremmende eigenschappen optreden.

Hoofdstuk 6 beschrijft veranderingen in circulerende serum CRP waarden, circulerende serum cholesterol waarden en plaque-kenmerken, invasief gemeten met IVUS en NIRS, na 12 maanden intensieve behandeling met rosuvastatine (de IBIS-3-studie). Bij patiënten met CAD vonden we klinisch relevante verlagingen van serum CRP-spiegels na 1 jaar intensieve (dagelijks 40 mg) rosuvastatine therapie. Deze veranderingen werden zowel bij de SAP als bij de ACS-patiënten waargenomen, maar waren het meest prominent bij ACS. Bovendien waren CRP-veranderingen gecorreleerd met veranderingen in d.m.v. IVUS gemeten plaque-kenmerken bij de ACS-patiënten. We vonden dergelijke correlaties niet bij de SAP-patiënten. Veranderingen in CRP waarden waren niet gecorreleerd met de waargenomen verlagingen in LDL-C. Vandaar dat onze studie de rol van ontsteking bij CAD-progressie ondersteunt, maar ook benadrukt dat de relatie tussen LDL-C-reductie, remming

van (vasculaire) ontsteking, veranderingen in omvang en samenstelling van coronaire plaques, en vermindering van het aantal coronaire 'events', niet eenvoudig is.

Hoofdstuk 7 beschrijft de implementatie van een elektronisch transmissie (e-transmissie) systeem voor elektrocardiogrammen (ECGs) in de regio Rotterdam. Buiten het ziekenhuis blijft de identificatie van patiënten met een acuut myocardinfarct (MI) een uitdaging in situaties waar het ECG geen specifieke ST-elevatie myocard infarct (STEMI) karakteristieken laat zien, en ook indien er andere onderliggende ritme- en geledingsproblemen zijn. De automatische ECG interpretatie geeft dan vaak een inconclusieve uitkomst. In december 2013 hebben we de geautomatiseerde ECG-apparaten van de ambulances in Rotterdam aangevuld met een modem, waardoor elektronische transmissie van de ECG's mogelijk werd. Het diagnostische protocol voor patiënten met acute ischemische pijn op de borst werd ook aangepast met nieuwe algoritmen. Alle ECGs met tekenen van een acuut myocard infarct (STEMI) werden doorgestuurd naar de interventie cardioloog en de patiënten werden aangekondigd in het PCI centrum. De overige ECGs werden bij twijfel doorgestuurd ter overleg met de interventie cardioloog. Tijdens de evaluatieperiode van 1 jaar, omvatte het studiecohort 1421 patiënten. Met toepassing van het gewijzigde pre-ziekenhuis triageprotocol ontvingen 115 patiënten met een acuut myocard infarct met een aanvankelijk niet doorslaggevend ECG een primaire PCI binnen 90 minuten, en 20 patiënten binnen 90-120 minuten. Aangezien onze studie geen gerandomiseerde studie is, kunnen we niet met volledige zekerheid concluderen dat de waargenomen vroege behandeling het directe gevolg is van een verandering in de patiëntenstroom die wordt veroorzaakt door het nieuwe triageprotocol. Desondanks hebben we op basis van de verkregen resultaten besloten het gebruik van het aangepaste protocol voort te zetten.

Deel II bloed biomarkers bij hartfalen

In het tweede deel van dit proefschrift genaamd "*bloed biomarkers bij hartfalen"* ligt de nadruk op de additionele waarde van bloed biomarkers bij patiënten met chronisch hartfalen (CHF) binnen de *"Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT*)" studie. De Bio-SHiFT studie is een prospectieve, observationele cohort studie van 263 klinisch stabiele patiënten met CHF opgezet in het Erasmus MC, Rotterdam, en de Noordwest Ziekenhuisgroep, Alkmaar. Patiënten met chronisch hartfalen, > 3 maanden geleden gediagnosticeerd volgens de richtlijnen van de 'European Society of Cardiology', werden benaderd tijdens hun regu-

liere poliklinische bezoeken in klinisch stabiele toestand. Bij aanvang en vervolgens om de 3 maanden (tot 30 maanden) werden bloed- en urine monsters verzameld en werd een korte medische evaluatie omtrent de klinische status (inclusief NYHA-klasse) gedaan. Door deze unieke studieopzet van herhaalde metingen waren we in staat om tijdens het proces van CHF het beloop van vele biomarkers te bestuderen. Deze dynamische patronen van de biomarkers werden vervolgens gebruikt om het risico van een patiënt op slechte klinische uitkomsten in te schatten.

Hoofdstuk 8 beschrijft de associaties tussen seriële metingen van N-terminal pro-brain natriuretic peptide (NT-proBNP), high sensitive troponine T (Hs-TnT) en C-reactive protein (CRP) met New York Heart Association (NYHA)-klasse, evenals met slechte klinische uitkomsten tijdens een mediane follow-up van 1 jaar. Temporele patronen van serieel gemeten NT-proBNP en Hs-TnT waren geassocieerd met herhaald vastgestelde NYHA-klasse. Daarnaast waren temporele patronen van NT-proBNP-, CRP- en NYHA-klasse onafhankelijk geassocieerd met slechte klinische uitkomsten (cardiovasculaire mortaliteit, ziekenhuisopname bij hartfalen, harttransplantatie en implantatie van Left Ventricular Assist Device (LVAD)). Seriële metingen van NT-proBNP en CRP verbeterden het onderscheidend vermogen bij het monitoren van CHF patiënten t.o.v. een model met alleen seriële NYHA-klasse metingen, hetgeen de waarde van seriële biomarkers metingen onderstreept.

Hoofdstuk 9 beschrijft de associatie tussen de dynamische, temporele patronen van serieel gemeten NT-proBNP, Hs-TnT en CRP met slechte klinische uitkomsten gedurende een follow-up verlengd tot een mediaan van 2.2 jaar. Met behulp van 'joint modeling' ontdekten we dat veranderingen in biomarker waarden, evenals de snelheid van de veranderingen (oftewel de helling van het temporele patroon) en langdurige verhogingen van gemeten NT-proBNP en CRP waarden,geassocieerd zijn met slechte klinische uitkomsten. Onze bevindingen suggereren dat het gebruik van individuele patronen van verandering van biomarker waarden, evenals de combinatie van meerdere biomarkers, klinisch relevant kunnen zijn bij het bepalen van de prognose van patiënten met stabiel CHF.

MicroRNA's (miR's) zijn nieuwe biomarkers die veelbelovend lijken voor vroege diagnostiek en behandeling van HF. MiR's zijn niet-coderende, ~ 22 nucleotide lange RNA-sequenties, die een rol spelen bij de splitsing of bij translationele repressie van messenger-RNA's en daardoor vele biologische processen beïnvloeden. **Hoofdstuk 10** beschrijft de associatie van seriële metingen van 7 MiR's die eerder gerelateerd werden aan HF (miR-1254, miR-22-3p, miR-423-5p, miR-486-5p en miR-320a) of verrijkt zijn in het myocard ,(miR-345-5p, miR-378a-3p) met slechte klinische

uitkomsten van patiënten met CHF. Het temporele patroon van circulerende miR-22-3p was geassocieerd met slechte klinische uitkomsten. Deze associatie was onafhankelijk van klinische karakteristieken van de patiënten en van temporele NT-proBNP-, HsTNT- en CRP-patronen. De snelheid van verandering in miR-22-3p-waarden was ook geassocieerd met klinische uitkomsten. Daarnaast waren temporele patronen van miR-22-3p geassocieerd met temporele patronen van HsTNT en CRP. Individuele patronen van circulerend miR-22-3p zouden dus nuttig kunnen zijn voor risicoprofilering bij CHF patiënten.

Hoewel bekend is dat renale dysfunctie vaak voorkomt bij patiënten met CHF, zijn de temporele evoluties van glomerulaire en tubulaire markers bij CHF nooit beschreven. **Hoofdstuk 11** toont aan dat bij patiënten met CHF de glomerulaire biomarkers (creatinine, geschatte glomerulaire filtratiesnelheid (eGFR) en cystatine C (CysC)), en tubulaire biomarkers (urinary N-acetyl-beta-D- glucosaminidase (NAG) en kidney-injured-molecule (KIM-1)) verslechteren tijdens progressie van CHF en dat ze slechte klinische uitkomsten voorspellen. Deze bevindingen zijn onafhankelijk van de klinische karakteristieken van een patiënt, farmacologische behandeling, cardiale natriuretische peptiden en troponines, en voor tubulaire markers is de voorspellende waarde ook onafhankelijk van eGFR. Voor zover wij weten, is dit de eerste studie die zowel de glomerulaire als de tubulaire functie tijdens langdurige follow-up van enkele jaren herhaaldelijk beoordeelt en daarmee aantoont dat beide renale compartimenten verslechteren tijdens progressie van HF, maar dat ze dit niet tegelijkertijd doen. De serumwaarden van renale biomarkers en ook de snelheid van de veranderingen die hierin optreden, zouden nuttig kunnen zijn voor dynamische risicoprofilering. Hierdoor kunnen artsen potentieel in staat worden gesteld om ziekte progressie te detecteren en om geïndividualiseerde behandelbeslissingen te nemen.

CONCLUSIES

De belangrijkste conclusies van het proefschrift kunnen als volgt worden samengevat:

Deel 1, bloed biomarkers en nieuwe beeldvormende technieken bij coronaire hartziekte:

- Een hogere SX-score is geassocieerd met een hogere mate van atherosclerose zoals beoordeeld door NIRS en RF-IVUS in een enkel niet-stenotisch kransslagadersegment bij patiënten met CAD.
- Een multiplex-panel van 26 inflammatoire biomarkers (acute-fase-eiwitten, cytokines en chemokines) en renale markers leverde geen bruikbare bloed biomarker op van hoge LCBI waarden gemeten met NIRS. Wel waren circulerende serum PCSK9-spiegels positief geassocieerd met hogere LCBI waarden gemeten met NIRS. Deze associatie was onafhankelijk van cardiale risicofactoren, evenals van serum LDL-C spiegels en statine gebruik.
- Plasma IL-8, plasma Cer (d18: 1/16: 0) en serum PCSK9-spiegels waren onafhankelijk geassocieerd met ongunstige cardiovasculaire uitkomsten tijdens een mediane follow-upperiode van 4.7 jaar bij patiënten met CAD.
- Na 1 jaar intensieve behandeling met rosuvastatine werden klinisch relevante verlagingen van CRP-spiegels waargenomen bij een reeks patiënten met bewezen CAD. De waargenomen CRP-veranderingen waren gecorreleerd met veranderingen in IVUS-afgeleide plaquekenmerken bij ACS-patiënten, maar niet bij SAP patiënten. CRP-veranderingen waren echter niet gecorreleerd met veranderingen in LDL-C-niveaus.

Deel II, Bloed biomarkers bij hartfalen:

- Serieel gemeten NT-proBNP en Hs-TnT waren positief geassocieerd met herhaald vastgestelde NYHA-klasse bij patiënten met stabiele CHF. Temporele patronen van serieel gemeten NT-proBNP, CRP en NYHA-klasse zijn sterk en onafhankelijk geassocieerd met slechte klinische uitkomsten in deze patiënten. Seriële metingen van NT-proBNP en CRP verbeterden het onderscheidend vermogen bij het monitoren van CHF patiënten t.o.v. een model met alleen seriële NYHA-klasse metingen. Dit benadrukt de waarde van biomarkers boven het alleen scoren van de NYHA klasse bij patiënten met CHF.
- De dynamische temporele patronen van serieel gemeten NT-proBNP en CRP levels zijn sterk en onafhankelijk geassocieerd met slechte klinische uitkomsten. De snelheid van de veranderingen in serumwaarden, en langdurige verhoging van de serumwaarden van deze biomarkers, zijn ook geassocieerd met slechte klinische uitkomsten.

- Het temporele patroon van circulerend miR-22-3p was onafhankelijk geassocieerd met slechte klinische uitkomsten en hierdoor een sterke voorspeller van de prognose bij CHF-patiënten.
- Serieel gemeten glomerulaire functie (creatinine, geschatte glomerulaire filtratiesnelheid (eGFR) en cystatine C (CysC)) en tubulaire functie (urinary N-acetyl-beta-D- glucosaminidase (NAG) en kidney-injured-molecule (KIM-1)) verslechteren, echter niet parallel aan elkaar, tijdens progressie van CHF, en voorspellen op dynamische wijze ongunstige klinische uitkomsten bij patiënten met CHF.

Klinische en toekomstige perspectieven

De visie op de pathofysiologie van coronaire hartziekte (CAD) heeft de afgelopen decennia een evolutie doorgemaakt. CAD wordt niet alleen gezien als een chronische, gestage en progressieve cholesterolstapelingsziekte maar als een multifactoriële, dynamische ziekte. CAD is complex en heterogeen van aard, omdat het wordt veroorzaakt door meerdere genetische, omgevings- en gedragsfactoren. Patiënten met verworven CAD moeten daarom op een geïndividualiseerde manier worden behandeld teneinde de kwaliteit van het leven te verbeteren, de sterftecijfers en ziekenhuisopnames te verminderen en om de kosten voor de gezondheidszorg te verlagen. Om een optimaal model te maken t.b.v. individuele risico-inschatting en behandeling, is het belangrijk om onze kennis te vergroten over de factoren die bijdragen aan een nadelig ziektebeloop bij deze patiënten. Dit kunnen we bereiken door verder onderzoek te doen naar de toegevoegde waarde van serum biomarkers en intracoronaire beeldvorming ten behoeve van het voorspellen van cardiovasculaire uitkomsten bij patiënten met verworven CAD.

Dit proefschrift toont aan dat invasieve beeldvorming van de kransslagaders door middel van RF-IVUS en NIRS bij patiënten met verworven CAD waardevolle informatie verschaft over de plaque-morfologie in het afgebeelde coronaire segment, en ingezet kan worden voor risicovoorspelling. Bovendien wordt aangetoond dat verschillende serum biomarkers geassocieerd zijn met ongunstige cardiovasculaire uitkomsten tijdens lange termijn follow-up. Het voordeel van bloed biomarkers is dat ze op een niet-invasieve manier en herhaaldelijk in de tijd gemeten kunnen worden. Verder onderzoek naar de waarde van herhaalde biomarker metingen is aangewezen. Studies die klinische kenmerken combineren met herhaalde biomarker metingen en met herhaalde intracoronaire beeldvorming zouden in de toekomst de individuele voorspellingen van een nadelig ziektebeloop kunnen verbeteren.

Bovendien toont dit proefschrift aan dat in patiënten met chronisch hartfalen, de tijdspatronen van meerdere circulerende biomarkers toegevoegde waarde hebben voor het voorspellen van de prognose. Hiermee hebben deze tijdspatronen potentie om het dynamische ziektebeloop nauwkeurig te volgen, wat nuttig zou kunnen zijn voor een geïndividualiseerde behandeling.

Dit proefschrift biedt hiermee een basis voor toekomstige, grotere studies die de waarde van tijdspatronen van deze biomarkers voor het bepalen van de prognose bevestigen, en die onderzoeken of de hier beschreven biomarkers gebruikt kunnen worden voor een biomarker-geleide behandelstrategie.

Hoewel de bevindingen van dit proefschrift veelbelovend zijn, is het essentieel dat ze worden bevestigd in grote, prospectieve, klinische studies. Ondanks dat er dus reeds veel vooruitgang is geboekt, blijft er nog steeds veel werk te verrichten in het veld van de gepersonaliseerde geneeskunde.

LIST OF PUBLICATIONS

Anroedh SS, Akkerhuis KM, Oemrawsingh RM, Garcia-Garcia HM, Brankovic M, Regar E, van Geuns RJ, Serruys PW, Daemen J, van Mieghem NM, Boersma E, Kardys I. Associations of 26 Circulating Inflammatory and Renal Biomarkers with Near-Infrared Spectroscopy and Long-term Cardiovascular Outcome in Patients Undergoing Coronary Angiography (ATHEROREMO-NIRS Substudy).
Curr Atheroscler Rep. 2018 Sep 14;20(10):52. doi: 10.1007/s11883-018-0752-8.
PMID: 30218437.

Anroedh S, Hilvo M, Akkerhuis KM, Kauhanen D, Koistinen K, Oemrawsingh R, Serruys P, van Geuns RJ, Boersma E, Laaksonen R, Kardys I. Plasma concentrations of molecular lipid species predict long-term clinical outcome in coronary artery disease patients.
J Lipid Res. 2018 Sep;59(9):1729-1737. doi: 10.1194/jlr.P081281. Epub 2018 Jun 1.PMID: 29858423.

Anroedh SS, Kardys I, Akkerhuis KM, Biekart M, van der Hulst B, Deddens GJ, Smits P, Gardien M, Dubois E, Zijlstra F, Boersma E. e-Transmission of ECGs for expert consultation results in improved triage and treatment of patients with acute ischaemic chest pain by ambulance paramedics.
Neth Heart J. 2018 Nov;26(11):562-571. doi: 10.1007/s12471-018-1187-0.
PMID: 30357611.

Vroegindewey MM, Schuurman AS, Kardys I, **Anroedh SS**, Oemrawsingh RM, Ligthart J, Garcia-Garcia HM, van Geuns RM, Regar E, Van Mieghem NM, Serruys PW, Boersma E, Akkerhuis M. SYNTAX score in relation to intravascular ultrasound and near-infrared spectroscopy for the assessment of atherosclerotic burden in patients with coronary artery disease.
EuroIntervention. 2019 Jan 20;14(13):1408-1415. doi: 10.4244/EIJ-D-17-00827.
PMID: 29537372.

Anroedh SS, Oemrawsingh RM, van Geuns RJ, Cheng JM, Garcia-Garcia HM, Daemen J, van Mieghem NM, Valgimigli M, Serruys PW, Boersma E, Kardys I*, Akkerhuis KM*. Association of serum PCSK9 with NIRS-derived lipid core burden index and longterm cardiac outcome. *Submitted*
* These authors contributed equally

Anroedh SS, Kardys I, Oemrawsingh RM, Lenzen MJ, van Geuns RJ, van Mieghem MJ, Daemen J, Ligthart JMR, Akkerhuis KM, Boersma E. Changes of C-reactive protein levels and coronary plaque composition after intensive statin therapy in patients with coronary artery disease. *Submitted*

van Boven N, Battes LC, Akkerhuis KM, Rizopoulos D, Caliskan K, **Anroedh SS**, Yassi W, Manintveld OC, Cornel JH, Constantinescu AA, Boersma E, Umans VA, Kardys I. Toward personalized risk assessment in patients with chronic heart failure: Detailed temporal patterns of NT-proBNP, troponin T, and CRP in the Bio-SHiFT study.
Am Heart J. 2018 Feb;196:36-48. doi: 10.1016/j.ahj.2017.10.008. Epub 2017 Oct 23.
PMID: 29421013.

van Boven N, Akkerhuis KM, **Anroedh SS**, Battes LC, Caliskan K, Yassi W, Manintveld OC, Cornel JH, Constantinescu AA, Boersma H, Umans VA, Kardys I. In search of an efficient strategy to monitor disease status of chronic heart failure outpatients: added value of blood biomarkers to clinical assessment.
Neth Heart J. 2017 Nov;25(11):634-642. doi: 10.1007/s12471-017-1040-x.
PMID: 28983818.

van Boven N, Akkerhuis KM, **Anroedh SS**, Rizopoulos D, Pinto Y, Battes LC, Hillege HL, Caliskan KC, Germans T, Manintveld OC, Cornel JH, Constantinescu AA, Boersma E, Umans VA, Kardys I. Serially measured circulating miR-22-3p is a biomarker for adverse clinical outcome in patients with chronic heart failure: The Bio-SHiFT study.
Int J Cardiol. 2017 May 15;235:124-132. doi: 10.1016/j.ijcard.2017.02.078. Epub 2017 Feb 22.
PMID: 28274577.

Brankovic M, Akkerhuis KM, van Boven N, **Anroedh S**, Constantinescu A, Caliskan K, Manintveld O, Cornel JH, Baart S, Rizopoulos D, Hillege H, Boersma E, Umans V, Kardys I. Patient-specific evolution of renal function in chronic heart failure patients dynamically predicts clinical outcome in the Bio-SHiFT study.
Kidney Int. 2018 Apr;93(4):952-960. doi: 10.1016/j.kint.2017.09.013. Epub 2017 Nov 28.
PMID: 29191357 Clinical Trial.

ABOUT THE AUTHOR

Sharda Suzan Anroedh was born on January 04, in Paramaribo, Suriname. After graduating high school in Suriname, she moved in 2006 to the Netherlands and started studying Medicine at the Erasmus University Rotterdam.

In 2012, she obtained the degree of medical doctor. Subsequently she started to work at the department of Internal Medicine in Vlietland ziekenhuis, Vlaardingen as a clinical resident. In 2014 she started as a PhD candidate at the department of Cardiology in Erasmus Medical Center, supervised by Prof. Dr. H. Boersma, Dr. I. Kardys and Dr. K.M. Akkerhuis. She was involved in prospective biomarker studies in acute coronary artery disease and in chronic heart failure. On top of that she was involved in the implementation of automated ECG devices on all ambulances in the Rotterdam-Rijnmond region. In 2017 she was awarded a Young Investigator Award in Atherosclerosis during the European Society of Cardiology Congress.

After fulltime work as PhD student, she started to work as clinical resident (ANIOS) at the department of Cardiology at Maasstad ziekenhuis, Rotterdam in 2018 to broad her vision and to obtain clinical experience. From August of 2019 she is working at the department of Cardiology at Hagaziekenhuis in Den Haag as clinical resident (ANIOS). Besides her work she enjoys travelling and spending time with her husband and daughter Parisha.

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PHD PORTFOLIO

PhD training	Year	Workload (ECTS)
Research skills		
Open Clinica Training	2014	0.3
Biomedical English Writing and Communication	2014	4.0
Biostatical Methods I: Basic Principles	2014	5.7
Pubmed Workshop	2014	0.3
Course Patient research	2014	0.6
Systematic literature search	2015	0.3
BROK (course on scientific integrity)	2017	1.5
In-depth courses		
COEUR-Cardiovascular Imaging and Diagnostics I,II and III	2014	4.5
COEUR-Vascular Clinical Epidemiology	2014	1.5
COEUR-Pathophysiology of ischemic heart disease	2014	1.5
COEUR-Congenital Heart Disease	2015	1.5
COEUR-Arrhythmia Research Methodology	2014	1.5
COEUR- Atherosclerotic and Aneurysmal Disease	2015	1.5
COEUR-Cardiovascular medicine	2015	1.5
COEUR-Heart Failure research	2016	1.5
COEUR-Atherosclerotic plaque imaging	2017	1.5
COEUR PhD days	2014-2017	1.2
Teaching activities		
Second year medical students: performing a systematic review	2014, 2015 and 2017	0.9
Presentations		
Journal Club (staflunch) 4x	2014-2017	1,2
Stichting Capri Hartrevalidatie Rotterdam (Cardiology group meeting Rotterdam-Rijnmond): 'Implementation of e-Transmission of ECGs.	2014	0.3
Maastad Ziekenhuis: 'Implementation of e-Transmission of ECGs.	2014	
Ambulance Rotterdam Rijnmond: 'Implementation of e-Transmission of ECGs. (4x)	2014-2016	1.2
ESC congress 2017, Barcelona (poster presentation (2X))	2017	0.6
ECS congress 2017, Barcelona (oral presentation)	2017	0.3
Congress		
ESC congress		1.5
NVVC Congress	2014,2015 and 2017	1.8

Blood biomarkers and novel imaging techniques in acquired heart disease

In this thesis, in patients with established CAD, the invasive coronary imaging modalities RF-IVUS and NIRS are shown to provide valuable information on plaque morphology in the imaged coronary segment and to be promising tools for risk prediction. Moreover, several circulating blood biomarkers are shown to be associated with adverse cardiovascular outcomes during long term follow-up. The major advantage of blood biomarkers is that they can be measured in a non-invasive manner and repeatedly over time. Further studies on the value of repeated biomarker measurements are warranted. In the future, studies combining clinical characteristics with repeated biomarker measurements and with repeated intracoronary imaging in a prognostic model may even further improve individual risk prediction. Such a prognostic tool may better identify 'high risk' patients and may thus facilitate clinical decision making.

In this thesis we also show that in patients with stable CHF, temporal patterns of several circulating biomarkers have incremental value for prognostication. Herewith these temporal patterns carry potential to accurately detect disease dynamics, which could be useful for tailored adjustment of treatment. This thesis provides a basis for future, larger studies to confirm the value of temporal patterns of these blood biomarkers for prognostication, as well as for studies on tailored, biomarker-guided adjustment of treatment strategy.