Advanced Methods for Clinical Outcome Prediction in Acquired Heart Disease

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Advanced Methods for Clinical Outcome Prediction in Acquired Heart Disease

Geavanceerde methoden voor klinische uitkomst voorspelling in verworven hart- en vaatziekten

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CONTENTS

PART I

Introduction

1

Introduction

1

Introduction

1

Introduction

Acquired heart disease, which includes conditions such as coronary artery disease (CAD) and heart failure, continues to pose a large impediment on the individuals that suffer from it as well as on society in general. CAD is the leading cause of death in the Western world, and the burden of CAD continues to rise in developing countries[1]. Heart failure is a chronic disease with frequent, costly re-hospitalizations[2]. The majority of heart failure cases results from CAD[1].

Overall, it has been estimated that by the year 2020, nearly 20.5 million deaths worldwide will be due to cardiovascular disease. Predictive thinking plays a fundamental role in prevention of adverse cardiac events, and the improvement of our ability to make accurate predictions is one of the driving forces behind clinical research. Focusing on patients with acquired heart disease for prevention of recurrent cardiac events and mortality may contribute to efficient healthcare, because these patients are at high risk of needing medical attention and may benefit most from additional treatment. This thesis overarches several disciplines, including methodology of prediction modelling, laboratory assessment and cardiovascular imaging, in order to evaluate and improve clinical outcome prediction in patients with known, acquired heart disease.

The purpose of this thesis was three-fold:

I. *To perform a critical appraisal of the methodology behind existing prediction models for both fatal and non-fatal adverse cardiac events in patients with CAD.*
   Multiple prediction models have previously been developed for patients with CAD, However, statements on a patient's individual prognosis remain challenging. We investigated the following research questions:

- Which are the limitations of existing prediction models?
- How could existing prediction models be improved?

II. *To investigate novel predictors for both fatal and non-fatal adverse cardiac events which may play a role in CAD.*
   Existing prediction models mostly use established risk factors. However, these do not explain a substantial proportion of adverse cardiac events. Inflammation has been recognized as an important pathophysiological mechanism contributing to CAD. The role of C-reactive protein in CAD had been examined extensively. Much less is known about the role of cytokines, chemokines and acute phase proteins, which are also involved in the inflammatory process. The research questions we examined were:

- Are cytokines, chemokines, and acute phase proteins associated with cardiovascular outcome?
- Are cytokines, chemokines, and acute phase proteins associated with the extent and composition of coronary atherosclerosis as assessed by intravascular ultrasound?

III. *To assess diagnostic and predictive value of biomarkers for adverse cardiac events in patients with heart failure, and for acute allograft rejection in patients who received a donor heart.*

Due to improved treatment of cardiac disease, patients currently survive longer after the initial manifestation of CAD. However, survivors are often left with damaged myocardium resulting in left ventricular dysfunction and, potentially, clinical heart failure. Blood biomarkers are potentially valuable tools in risk stratification of these patients. The following research questions were defined:

- Which biomarkers play a role in patients with heart failure with normal ejection fraction?
- Are repeatedly measured NT-proBNP, cardiac troponin T and/or CRP associated with acute allograft rejection in heart transplant recipients?

The outline of this thesis is as follows. **Part II** focuses on prediction models of both fatal and non-fatal adverse cardiac events in patients with established, stable CAD. An overview of the existing prognostic models for the prediction of fatal endpoints in patients with established, stable CAD is presented in **Chapter 2**. We discuss the strengths and weaknesses that underlie the statistical analyses which were applied to assess model performance. Furthermore, we develop and validate a series of risk prediction models for both fatal and non-fatal endpoints in a large prospective cohort of European patients with established CAD, making use of the EUROPA database[3]. In **Chapter 3,** we present an alternative for clinical decision-making in individual patients, so-called micro-simulation [4]. Micro-simulation replicates individual patient histories, and may thus inform physicians by estimating the most likely outcomes regarding a broad range of clinical events. Subsequently, we develop a new micro-simulation software package which is applicable for prognostic modeling in patients with established CAD.

Part III focuses on inflammatory agents for the prediction of cardiovascular outcome[5], as well as the extent and composition of coronary atherosclerosis in patients with acquired heart disease [6, 7]. In **Chapter 4** we describe several signaling cascades of biomarkers implicated in the pathogenesis of atherosclerosis, and the utility of these biomarkers to improve the prediction of clinical events, and the extent and vulnerability of coronary atherosclerosis as determined by virtual histology (VH)- intravascular ultrasound (IVUS) [8]. In this context, we consecutively evaluate cytokines, chemokines, and acute phase proteins. In **Chapter 5,** we study whether there are differences in extent and composition of coronary atherosclerosis between smokers and non-smokers.

In **Part IV,** we provide an overview of blood biomarkers that have been found to be associated with the occurrence and prognosis of heart failure with normal ejection fraction (**Chapter 6**). Furthermore, we examine diagnostic and predictive value of the biomarkers NT-pro-B-

type natriuretic peptide (NT-proBNP), cardiac troponin T and C-reactive protein (CRP) for allograft rejection in patients that have undergone heart transplantation(**Chapter 7**). In examining predictive value, we apply sophisticated methods for repeated measurements data analysis known as joint modelling [9].

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PART II |

Methodological aspects of prediction models for clinical outcome in coronary artery disease

21

Appraisal of state-of-the-art prediction models

12

Appraisal of state-of-the-art prediction models

2.1|

Overview of existing prediction models

Linda Battes, K. Martijn Akkerhuis, Nick van Boven,
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ABSTRACT

Background: Installment of appropriate measures to prevent adverse events in patients with established, stable coronary artery disease (CAD) may contribute to efficient healthcare. This review gives an overview of existing models for prediction of cardiovascular adverse events in such patients and discusses model performance.

Methods: We used a computerized literature search in the EMBASE, PubMed publisher, MEDLINE, Google Scolar, Web of Science and Cochrane databases. Studies were selected if they included patients with stable CAD (stable angina pectoris, myocardial infarction more than 3 months ago or coronary intervention more than 6 months ago) and if they presented a model that included mortality as the endpoint.

Results: Sixteen studies met our inclusion criteria. Clinical variables that were included in the models differed highly between the studies. Still, age, smoking status, hypertension, diabetes, cholesterol and heart failure were present in a large part of the models. Several studies examined model discrimination, but the majority paid insufficient attention to calibration and validation.

Conclusions: Although multiple prediction models for adverse events have been developed in patients with stable CAD, variables included in these models display large heterogeneity, and model performance is often insufficiently addressed.

INTRODUCTION

Coronary artery disease (CAD) is the leading cause of death in the Western world, and the burden of CAD continues to rise in developing countries(1). It has been estimated that by the year 2020, nearly 20.5 million deaths worldwide will be due to cardiovascular disease (1). This trend may be altered by installing additional preventive measures. For this purpose, appropriate risk stratification tools should be available. As such, a large number of risk prediction models and risk charts have been developed in the field of cardiovascular disease, both for primary prevention and for secondary prevention (2-4).

Secondary prevention, or prevention of mortality and recurrent events in patients with established CAD, may contribute to efficient healthcare, because these patients are at risk of needing medical attention and may benefit most from additional treatment (5). Appropriate risk stratification in this population may identify patients that could be followed-up more closely, may be treated more aggressively, and whose compliance to prescribed drugs may be monitored more carefully, in order to prevent recurrent events. Furthermore, patient awareness of the magnitude of their risk for having a recurrent event might enhance their compliance to prescribed medication (6) and may also stimulate lifestyle changes (6).Therefore, there is a great need for adequate prediction models that assess long-term risk of adverse cardiovascular events in patients with stable CAD. Such models, designed specifically for stable patients with established CAD are scare.

The purpose of this review is to give an overview of the existing prognostic models for the prediction of sudden cardiac death, cardiovascular mortality and all-cause mortality in patients with established, stable CAD. An overview of model performance will be given. Moreover, the strengths and weaknesses that underlie the statistical analyses which were applied to assess model performance will be addressed.

METHODS

On 1 September 2013 we performed a literature search using EMBASE, PubMed publisher, MEDLINE, Google Scolar, Web of Science and Cochrane for studies which included patients with stable, established coronary artery disease and developed a prediction model or risk stratification model for long-term predictions of sudden cardiac death, cardiovascular mortality, and all-cause mortality. We defined patients who had stable coronary artery disease at inclusion as those that had stable angina pectoris, those that had experienced a myocardial infarction more than 3 months ago, or those that had undergone a percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) more than 6 months ago(7, 8). Only studies which examined one or more of the endpoints sudden cardiac death, cardiovascular mortality, all-cause mortality, or mortality in combination with one or more non-fatal cardiovascular endpoints, were selected. We limited our search to

articles written in English. The search strategy is described in detail in the appendix. A total of 3672 articles were obtained, and their titles and abstracts were screened for relevance. Another 3 articles were added by searching the reference lists for relevant articles,
Based on title and abstract, 202 articles were selected that apparently met our inclusion criteria. Articles were subsequently excluded: when the study included patients with merely suspected CAD, alone or in combination with established CAD; when the main outcome was limited to short-term (in-hospital) mortality only; when patients with LVEF < 40% or arrhythmias were included; when only patients with specific cardiovascular risk factors such as hypertension or diabetes were selected; or when the research question was limited to the prognostic value of a specific variable (e.g., a blood biomarker), without presentation of a complete risk stratification model. After reading the full articles, 16 publications remained, and were included in this review (figure 1).

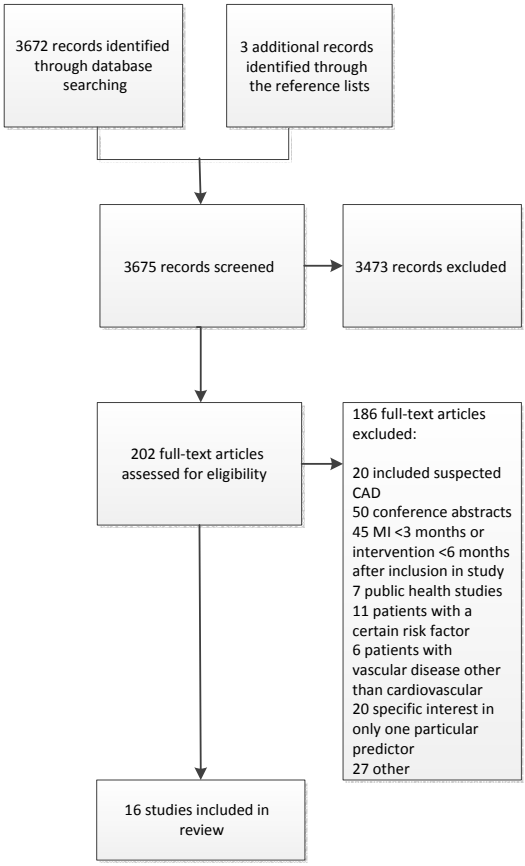

Figure 1. Flowchart showing the number of abstracts identified by the search, the number of full texts which remained, and the number of studies included in the review.

RESULTS

A total of 16 risk prediction models were found. Sample sizes were generally large, ranging up to 37,258 (9) patients, with a mere 4 studies consisting of less than 1000 patients (10-13). Table 1 shows the inclusion criteria, the mean follow-up time and the primary endpoints of the included studies.

Study design

Of the 16 studies we found, one study collected data retrospectively from medical records (14). One study was designed as a survey analysis(15). Fourteen studies had a prospective design (9-13, 16-24), eight of which were clinical trials. These clinical trials tested the effectiveness of perindopril versus placebo (16, 17), pravastatin versus placebo (20), atenolol, nifedipine or a combination of both(13), trandolapril versus placebo(21), nicorandil versus placebo(22), atorvastatin versus placebo(23) and nifedipine versus placebo(24).

Patient population

Four studies included patients with clinically diagnosed stable angina pectoris (13, 19, 22, 24). Two studies included patients with stable angina pectoris as well as a certain degree of stenosis on angiography (9, 18). Two studies included patients with a confirmed diagnoses of MI in the last 3 years(11, 20), and one study included patients 5 years after a CABG (10). The remaining studies used various combinations of criteria to define established CAD (12, 14-17, 21, 23).

Outcomes

Most of the studies aimed to predict the risk of cardiovascular mortality(10, 14-16, 20, 23), all-cause mortality (11, 12, 24), or sudden cardiac death(9, 21) with a median follow-up duration after inclusion up to 11.3 years(12). Some of these studies additionally examined separate, non-fatal outcomes such as MI or stroke (11, 16, 20, 24). One study used the combined endpoint of all-cause mortality and MI (19), and five studies uses the combined endpoint cardiovascular mortality and MI (10, 13, 17, 18, 22). The incidence rates of the endpoints which were examined ranged between 0.3 (21) and 13.8 (11) percent per year (table 1)

Table 1. Risk stratification models: patients, follow-up time and endpoints

Title	Author, year	Patients	No. of patients	Follow-up duration	Endpoints	Incidence rate (% per year)‡	Study type
Stress cardiac single-photon emission computed tomographic imaging late after coronary artery bypass surgery for risk stratification and estimation of time to cardiac events(10)	Acampa, 2008	Patients 5 years after CABG#	362	median 27 months	Combined endpoint: cardiac death or MI**	2.7	prospective
Prognostic factors in patients who have survived myocardial infarction(11)	Aleksic, 2010	Confirmed diagnoses of MI** in the last 3 years	118	mean 3 years	Combined endpoint: all-cause mortality, revascularization or reinfarction	13.8	prospective
Usefulness of the Duke Sudden Cardiac Death Risk Score for Predicting SCD in patients with angiographic (>75% narrowing) coronary artery disease(9)	Atwater, 2009	Patients with at least one native cronary artery stenosis of ≥ 75%	37258	median 6.2 years	Sudden cardiac death	0.7	prospective
Development and validation of a cardiovascular risk assessment model in patients with established coronary artery disease(16)	Battes, 2013	Established CAD+ (prior MI** (>3 months ago), or prior PCI++/CABG# (>6 months ago) or angiographic diameter stenosis ≥ 70%)	12218	median 4.1 years	Cardiac death, noncardiac death, nonfatal MI**, CABG#, PCI++, resuscitated cardiac arrest, and combinations of these endpoints	Cardiac death: 0.9	prospective
Risk score for predicting death, myocardial infarction, and stroke in patients with stable angina, based on a large randomized trial cohort of patients(24)	Clayton, 2005	Patients with stable symptomatic angina requiring treatment and either previous MI** or proved angiographic CAD+. Or a positive result on an exercise or perfusion test	7311	mean 4.9 years	All-cause mortality, MI**, or stroke	All-cause mortality: 1.6	clinical trial
Laboratory and non-laboratory-based risk prediction models for secondary prevention of cardiovascular disease: the LIPID study(20)	Cui, 2009	Patients who had an acute MI** or were hospitalized for unstable AP- within the previous 3 months to 3 years	8557	median 6 years	Combined endpoint: MI**, stroke or cardiac death	2.9	clinical trial

Table 1. Continued

Title	Author, year	Patients	No. of patients	Follow-up duration	Endpoints	Incidence rate (% per year)‡	Study type
The value of routine non-invasive tests to predict clinical outcome in stable angina(13)	Daly, 2002	Stable AP- and a positive exercise test	682	mean 2 years	Combined endpoint: unstable AP-, MI** or cardiac mortality	5.2	clinical trial
Predicting prognosis in stable angina - results from the Euro heart survey of stable angina: prospective observational study(19)	Daly, 2006	Patients with new presentation of stable AP- and consecutive patients with clinical diagnosis of stable AP- caused by myocardial ischaemia due to coronary disease	3031	median 1.1 years	Combined endpoint:all-cause mortality or non-fatal MI**	2.8	prospective
Residual risk of cardiovascular mortality in patients with coronary heart disease: The EUROASPIRE Risk Categories(14)	De Bacquer, 2012	CABG#, PCI++, acute MI**, myocardial ischemia were included at least 6 months after their acute event or procedure	5216	median 4.6 years	Cardiac death	1.4	retrospective
Treatment benefit by perindopril in patients with stable coronary artery disease at different levels of risk(17)	Deckers, 2006	Established CAD+ (prior MI** (>3 months ago), or prior PCI++/CABG# (>6 months ago) or angiographic diameter stenosis ≥ 70%)	12218	median 4.1 years	Combined endpoint: cardiac death or nonfatal MI**	2.2	prospective
Routinely available biomarkers improve prediction of long-term mortality in stable coronary artery disease, the Vienna and Ludwigshafen Coronary Artery Disease (VILCAD) risk score(12)	Goliasch, 2012	Established CAD+ (angiographical evidence of stenosis of an epicardial coronary artery of ≥ 60%)	547	median 11.3 years	All-cause mortality	3.4	prospective

Table 1. Continued

Title	Author, year	Patients	No. of patients	Follow-up duration	Endpoints	Incidence rate (% per year)‡	Study type
Sudden cardiac death in patients with stable coronary artery disease and preserved left ventricular systolic function(21)	Hsia, 2008	Angiographic diameter stenosis > 50%, prior PCI++/CABG#, prior MI**, preserved LVEF## >40% or qualitatively normal left ventriculogram or the absence of wall motion abnormality on echocardiogram.	8290	median 4.8 years	Sudden cardiac death	0.3	clinical trial
Benefit of adding lifestyle-related risk factors for prediction of cardiovascular death among cardiac patients(15)	Ingle, 2013	CAD+ patients from the Health Survey for England and Scottish Health Survey	1372	mean 7 years	Cardiac death	2.1	survey analysis
Determinants of coronary events in patients with stable angina: Results from the Impact of Nicorandil in Angina Study(22)	McMahon, 2005	Patients with a history of AP-	5047	mean 1.6 years	Combined endpoint: cardiac death or nonfatal MI**	8.9	clinical trial
Determinants of residual risk in secondary prevention patients treated with high- versus low-dose statin therapy(23)	Mora, 2012	Established CAD (previous MI**, previous or present AP- with objective evidence of atherosclerotic coronary disease, or previous coronary revascularization procedure)	9251	median 4.9 years	Combined endpoint: cardiac death, nonfatal MI**, resuscitation after cardiac arrest, fatal or nonfatal stroke	1.6	clinical trial
Multiple marker approach to risk stratification in patients with stable coronary artery disease(18)	Schnabel, 2010	Patients with manifest CAD+, with at least one stenosis ≥ 30% in a major coronary artery	1781	mean 3.6 years	Combined endpoint: cardiac death or non fatal MI**	2.1	prospective

‡Incidence rate approximated by dividing no. of events reported in paper by (no. of persons x mean or median follow-up duration reported in paper) and presented in % per year; # CABG= Coronary Artery Bypass Grafting ; **MI = Myocardial Infarction; + CAD = Coronary Artery Disease; - AP= Angina Pectoris; ++ PCI= Percutaneous Coronary Intervention; ##LVEF=Left Ventricular Ejection Fraction

Variable selection and content of ensuing models

Table 2 displays the variables included in the 16 prediction models. All studies applied Cox proportional hazard regression. Variables contributing to the prediction of the endpoint(s) were selected using the stepwise backward method (11, 14, 16, 17, 21), or the stepwise forward method(9, 10, 13, 22-24), and two studies applied both methods(12, 19). Schnabel et al. (18) did not use a variable selection procedure; they constructed their model from the ESC SCORE risk factors. Ingle et al. (15) did not use a variable selection procedure either; they constructed their model from the Framingham risk factors and included further novel predictors. Cui et al. (20) considered a variety of risk models with and without laboratory data without using variable selection.

Only gender (12, 14-16, 22) or both age and gender (9, 10, 13, 19) were absent in many of the risk prediction models we reviewed. Presence of the clinical variables age (11, 12, 14-18, 20-24), diabetes (9, 10, 12, 14-20, 23, 24), hypertension (9, 17, 18, 20, 23, 24), smoking (9, 14-18, 20, 22, 23), cholesterol (11, 14-18), heart failure (9, 12, 13, 16, 19, 21, 24), and heart rate (12, 22) in the models differed among the studies.

Several studies included routinely used laboratory biomarkers for long-term prediction. Ingle et al. (15) examined whether addition of CRP to clinical variables improved model discrimination. Goliasch et al. (12) hypothesized that biomarkers representing renal and liver functions might be useful to add to the prediction model, because these may be influenced by the set of drugs that CAD patients receive. Of these biomarkers, only cholinesterase was an independent predictor for all-cause mortality. Schnabel et al.(18) examined the prognostic ability of 12 biomarkers in the prediction of cardiovascular endpoints. They used classical cardiovascular risk factors to derive the primary risk categories, and assessed the added value of the selected biomarkers. They showed that adding NT-proBNP, GDF-15, MR-proANP, cystatin C, and MR-proADM to the models provided incremental value. Mora et al. (23) investigated determinants of risk in the Treating to New Targets (TNT) study, a clinical trial that randomized patients to atorvastatin 80 or 10 mg/d. They concluded that high-dose statin use and baseline apolipoprotein A-I were determinants of decreased risk for cardiovascular events, while baseline apolipoprotein B and blood urea nitrogen were determinants of increased risk.

Clayton et al. (24) included modifiable, procedural, and laboratory variables in their multivariate analysis. Of the laboratory variables, white blood cell count, glucose and creatinine were independent predictors of the combined endpoint all-cause mortality, MI, or stroke.

Eight studies developed risk scores based on the prediction models they described (table 3). These risk scores were usually based on points systems, and could serve as a means for making the above-described risk prediction models useful to clinicians.

Table 2. Overview of variables included in the prediction models resulting from the studies.

Variable	STUDY															
	Acampa, 2008	Aleksic, 2010*	Atwater, 2009	Battes, 2013	Clayton, 2005	Cui, 2009	Daly, 2002	Daly, 2006	DeBacquer, 2012	Deckers, 2006	Goliasch, 2012	Hsia, 2008	Ingle, 2013	McMahon, 2005	Mora, 2012	Schnabel, 2010
Age	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1
Gender	0	1	0	0	1	1	0	0	0	1	0	1	0	0	1	1
Race	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Hypertension	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1	1
Heart rate	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Smoking status	0	0	1	1	0	1	0	0	1	1	0	0	1	1	1	1
Diabetes Mellitus/ blood glucose	1	0	1	1	1	1	0	1	1	1	1	0	1	0	1	1
Cholesterol	0	1	0	1	0	0	0	0	1	1	0	0	1	0	0	1
BMI[+]	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1
Peripheral vessel disease	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Angina Pectoris	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0
Prior CHD/revascularization	0	0	0	1	1	1	1	0	0	1	0	0	0	1	1	0
Prior stroke/TIA	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0
Congestive heart failure/LVEF[++]	0	0	1	1	1	0	1	1	0	0	1	1	0	0	0	0
Renal disease/renal function	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0
Family history CAD	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Comorbidity	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Cardiac medication use	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	0
Laboratory findings	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	1
Priority PCI[%] /CABG[*]	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
Multivessel disease/ no. of diseased vessels	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1
ECG findings [#]	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0
Ischemia/ perfusion defects at SPECT	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Physical activity	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Change number of risk factors	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exercise test results/ST segment changes	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Echographic findings	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0

[+] BMI = Body Mass Index; [++] LVEF = Left Ventricular Ejection Fraction; [%] = PCI = Percutaneous Coronary Intervention; [*] = CABG= Coronary Artery Bypass Grafting; [#] ECG = electrocardiography

Performance assessment

Performance assessment of risk stratification models and prediction models is essential, since they are meant to support decision making in clinical practice or to provide prognostic estimates (25). Several methods are available to examine model performance

and to evaluate the quality of the predictions (26), including measures of discrimination and calibration as well as overall performance measures. Table 3 displays the various performance measures which were used in the studies we reviewed.

Calibration

The Hosmer-Lemeshow test is one of the traditional performance measures which compares observed to predicted outcome, often by deciles of predicted probability, thus testing calibration. Only 6 of the 16 studies described model calibration. Atwater et al.(9), Battes et al.(16), Clayton et al. (24), Cui et al. (20), Deckers et al.(17), and Mora et al. (23) used the Hosmer-Lemeshow plot. In all six studies the agreement between observed and predicted mortality rates was adequate.

Discrimination

Discrimination refers to the ability of a model to discriminate between those with and those without the outcome of interest. Calculating the concordance (c) statistic or the area under the receiver operating characteristic (ROC) curve (AUC) is the method which is applied most often for this objective(26). Ten of the 16 studies used this method, the remaining six studies did not quantify the discriminative abilities of their model. Cui et al.(20), Goliasch et al.(12), Mora et al.(23), and Daly et al. (19) used the Harrell's C-statistic, which approximates the AUC. (27) C statistics ranged from 0.66 for the model of Cui et al.(20), which predicted cardiovascular mortality, MI or stroke in patients who had an acute MI or were hospitalized for unstable AP in the last 3 years (n=5654), to 0.77 for the model of Goliasch et al. (12) which predicted all-cause mortality in patients with stable CAD (n=547) defined as angiographical evidence of stenosis of an epicardial coronary artery of ≥ 60% in the derivation cohort and of ≥ 50% in the validation cohort.

Four models, those of Cui et al.(20), Schnabel et al. (18), Deckers et al.(17), and Mora et al.(23), displayed c statistics below 0.70, which was somewhat lower than those of the six other studies. The study of Cui et al. (20) considered 6 models with and without laboratory-based variables. The models were constructed using selected variables which are generally used in risk models meant for primary prevention, without using stepwise routines. The model of Schnabel et al.(18) was constructed based on variables from the ESC SCORE (2). This score, on its part, was based on data from the general population, not data from patients with pre-existing CAD. These results imply that the ESC SCORE, as well as variables derived from other primary prevention risk scores, may not discriminate well in CAD patients. On the other hand, it should be noted that model performance assessment in datasets which are also used for model development, may provide overly optimistic performance estimates because of overfitting (28). Deckers et al.(17) and Battes et al.(16) both developed their prediction models making use of the EUROPA dataset(29).

While Deckers et al.(17) focused on a combined endpoint which comprised fatal as well as nonfatal cardiovascular adverse events, Battes et al.(16) examined additional cardiovascular endpoints, both fatal and nonfatal as well as combined. The latter analyses demonstrated that discrimination with regard to fatal endpoints was good, while discrimination for nonfatal endpoints was poor. The latter held up when nonfatal endpoints were combined with fatal endpoints into a composite outcome. These results suggest that, when modeling prognosis in patients with stable CAD, pooling several outcomes may influence model performance to a great extent and caution is needed when examining nonfatal endpoints or combined outcomes that include such end points. In parallel, Mora et al. (23) used a combined endpoint which included coronary death, non-fatal MI, resuscitation after cardiac arrest, and fatal or non-fatal stroke.

Overall performance measures

The distance between the predicted outcome and actual outcome is central to quantify overall model performance from a statistical perspective. This distance is related to the concept of "goodness-of-fit" of a model, with better models having smaller distances between predicted and observed outcome (30), and may be assessed by using measures such as the amount of explained variation (R^2), Nagelkerke's R^2, (log) likelihood ratio, Bayes information criterion (BIC), Akaike information criterion (AIC), or the Brier score. Battes et al.(16) tested the overall performance of their model using Nagelkerke R^2, which was good for cardiovascular mortality (12%). Several studies used the (log) likelihood ratio during model development (9, 14, 18, 23). Cui et al.(20) examined both AIC and BIC to select the best fitting model.

Reclassification

To quantify improvement in model performance introduced by adding new variables to the existing model, net reclassification improvement (NRI) can be calculated (31). For this purpose, patients that have experienced an event are divided into categories of low-, intermediate- and high-risk, and the same is done in patients that have not experienced an event. This division in categories is done with and without the addition of the new marker. The correct movement in categories - upwards for events and downwards for non-events - can subsequently be quantified. Only Schnabel et al. (18) applied this method. They examined the prognostic ability of 12 biomarkers in the prediction of cardiovascular endpoints, using classical cardiovascular risk factors to derive the primary risk categories, and assessed the added value of the selected biomarkers.

Table 3. Performance assessment.

Author/ year	Calibration		Discrimination	Overall performance measures				Reclassification		Type of validation		Risk score
	Calibration plot	HL test*	C-statistic	R^2	(Log) likelihood	AIC#	BIC ##	NRI**	IDI+	Internal	External	Yes/ no
Acampa, 2008	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	no
Aleksic, 2010	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	no
Atwater, 2009	1	1	c= 0.75	NA	1	NA	NA	NA	NA	bootstrapping	1	yes
Battes, 2013	1	NA	c=0.73	1	NA	NA	NA	NA	NA	split-sample	NA	no
Clayton, 2005	1	NA	NA	NA	NA	NA	NA	NA	NA	bootstrapping	NA	no
Cui, 2009	NA	1	c=0.66	NA	NA	1	1	NA	NA	NA	1	yes
Daly, 2002	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	yes
Daly, 2006	NA	NA	c= 0.74	NA	NA	NA	NA	NA	NA	NA	NA	no
De Bacquer,2012	NA	NA	NA	NA	1	NA	NA	NA	NA	NA	NA	yes
Deckers, 2006	1	NA	c= 0.67	NA	NA	NA	NA	NA	NA	NA	NA	yes
Goliasch, 2012	NA	NA	c=0.77	NA	NA	NA	NA	NA	NA	bootstrapping	1	yes
Hsia, 2008	NA	NA	c= 0.71	NA	NA	NA	NA	NA	NA	cross-validation	NA	yes
Ingle, 2013	NA	NA	c=0.75	NA	NA	NA	NA	NA	NA	NA	NA	no
McMahon, 2005	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	no
Mora, 2012	NA	1	c= 0.68	NA	1	NA	NA	NA	NA	NA	NA	no
Schnabel, 2010	NA	NA	c= 0.69	NA	1	NA	NA	GDF-15: 0.16 NT-proBNP: 0.15 MR-proANP: 0.13 Cystatin C: 0.12 MR-proADM: <0.1	GDF-15: 0.021 NT-proBNP: 0.022 MR-proANP: 0.014 Cystatin C: 0.015 MR-proADM: 0.022	NA	NA	yes

* HL-test = Hosmer-Lemeshow test; #AIC = Akaike information criterion, ## = Bayes information criterion, ** NRI = net reclassification index; + IDI = integrated discrimination improvement; ++ NA = not applicable; 1= test performed

The strongest reclassification improvement for single biomarkers, with a NRI of over 10%, was observed for GDF-15, NT-proBNP, MR-proANP and cystatin C. A related method to assess improvement in risk stratification models is integrated discrimination improvement (IDI), which uses differences between integrated sensitivities and 'one minus specificities' for models with and without the new marker (26). Schnabel et al.(18) found an IDI for the combination of biomarkers GDF-15, NT-proBNP, MR-proANP, MR-proADM and cystatin C of 0.04 ($p<0.0001$).

Validation

Internal validation assesses validity for the setting from which the data originated (30). Original data may be split into a testing and training set; models may be developed in the testing set and may subsequently be validated in the training set. Techniques to obtain testing and training sets include split-sample, cross-validation, and bootstrapping (32) methods. Bootstrapping is advantageous is terms of statistical power. It replicates the process of sample generation from an underlying population by drawing samples with replacement from the original data set, of the same size as the original data set (32). External validity, or generalizability, refers to assessment of model performance in patients from a different but "plausibly related" population (28). External validation enables investigation of general applicability of the prediction model.

Testing the validity of a model is essential to assess its reliability. Internal validation was done by five studies. Atwater et al. (9), Goliasch et al. (12), and Clayton et al. (24) used bootstrapping, Battes et al.(16) used split-sample and Hsia et al.(21) used cross-validation. In none of the studies, internal validation pointed to overoptimism in the final model's discrimination. In three of the models we reviewed, external validity was assessed. Atwater et al.(9) externally validated their risk score in patients with ischemic cardiomyopathy, with a moderate c-index of 0.64, compared to 0.75 on internal validation. Cui et al.(20) developed their models based on data from individuals recruited in Australia, and validated these models using data from individuals recruited according to a similar protocol in New Zealand. After recalibration, the models displayed a good fit (Hosmer-Lemeshow test: $p=0.42$). Goliasch et al.(12) validated their risk score in the Ludwigshafen Risk and Cardiovascular Health study and achieved similar results as those in their original dataset (c-index= 0.73, $p <0.001$).

DISCUSSION

In this review, we have summarized 16 risk prediction models for patients with stable coronary artery disease by reviewing their content and their performance. In general, the sample sizes of the studies we reviewed were large, ranging up to 37.258 (9) patients, and

heterogeneous, ranging from patients with stable angina to patients who experienced MI in the past. All studies in this review used outcomes that included cardiovascular mortality and/or all-cause mortality. Notably, incidence rates of the outcomes that were examined varied substantially across the studies. Overall, the variables that were available for multivariable model building in the various studies were highly similar. The majority of the studies had data available on established cardiovascular risk factors (including laboratory variables like cholesterol or renal function), with the exception of Daly et al. (13), who were particularly interested in the value of routine non-invasive tests as predictors. Nevertheless, the variables which were eventually included in the prediction models differed largely between the studies; there were no predictors which were included in all of the models. Many prediction models included established cardiovascular risk factors; four studies (17, 18, 20, 23) included at least age, diabetes, hypertension and smoking. Furthermore, several models contained the variables cholesterol (11, 14-18), heart failure (9, 12, 13, 16, 19, 21, 24), and heart rate (12, 22). Five studies also included laboratory findings in addition to clinical variables to their final model. Overall, model performance, including calibration and discrimination, was not examined extensively in the studies.

Currently, use of risk prediction models is widely accepted in the field of primary prevention of cardiovascular disease. One of the models that is frequently used is the Framingham risk score (33). The variables in this score are age, sex, blood pressure, total cholesterol, high-density lipoprotein (HDL), cigarette smoking and diabetes. Recently, the clinical significance of the age-adjusted Framingham risk score for secondary prevention was evaluated by Park et al.(34). They investigated the predictive power of this score for incident coronary events in patients with stable angina pectoris, without a history of ACS. The AUC was 0.863, which indicates adequate discrimination. However, the study population was small (n =71), and elaborate exclusion criteria were applied, resulting in inclusion of relatively healthy patients. Therefore, the reliability of this optimistic estimate of the AUC may be questioned. The Framingham risk score has also been used by Ingle et al.(15) to examine the predictive value of CRP or lifestyle related variables (including moderate-to-vigorous physical activity, BMI and psychological distress) on top of this score in 1372 patients with pre-existing CHD from the Health Survey for England and Scottish Health Survey. The AUCs of this risk score with addition of CRP for the endpoint CVD death were 0.73 for males and 0.74 for females, respectively. In this study, calibration measures were also missing. These results illustrate that additional research is warranted to examine the generalizabilty of the Framingham risk score to patients with a pre-existing CHD.

Remarkably, only gender(12, 14-16, 22) or both age and gender (9, 10, 13, 19) were absent in many of the risk prediction models we reviewed. Gerber et al. (35) investigated

the influence of gender differences on the role of classic risk factors in the prediction of recurrent ischemic events after MI. Hypertension and diabetes were stronger predictors in women than in men for having a recurrent event. Hypercholesterolemia was associated with recurrent events only in women. Furthermore, a recent study of Lehto et al. (36) examined the difference in CVD risk between men and women from a general population according to different age groups. In the age group younger than 55, CVD risk was similar in both genders, probably due to the high prevalence of diabetes in relatively young women, while men had a higher CVD risk than women in the age group greater than 55, due to smoking, hypertension and history of a prior CVD event. These findings illustrate that the association of classic risk factors with the risk of having recurrent ischemic events differs by gender and age, and demonstrate the potential importance of these risk factors for prediction models.

In general, large variation was present in classic cardiovascular risk factors, other than age and gender, which were included in the different models. Part of this variation may probably be explained by differences in the study populations and in the incidence rates. Furthermore, the variation might also in part be due to differences in prevalence of risk factors and in use of cardioprotective medication between different countries (37). EUROASPIRE III (37), for example, showed that a large number of patients with CAD do not reach the lifestyle and therapeutic targets for cardiovascular disease prevention according to the guidelines. These differences may play a part in the selection of the variables in the various prediction models.

The majority of prediction models we reviewed included diabetes. Fox et al. (38) have shown that the proportion of cardiovascular disease due to diabetes has drastically increased over the past 50 years. It has been demonstrated that after a cardiovascular event has occurred, risk of recurrent events stays high in diabetic patients (39). Furthermore, diabetes is often associated with other cardiovascular risk factors like hypertension (39). Of the studies we reviewed, twelve included diabetes in their prediction model (9, 10, 12, 14-20, 23, 24), and of these studies six included hypertension as well (9, 17, 18, 20, 23, 24), which is in agreement with previous findings from the literature. The remaining studies did not include diabetes in their final model because of a non-significant result after running the multivariable analysis (11, 13, 21, 22).

Some aspects of our review warrant further consideration. The definition of stable CAD was somewhat different between the various studies. We included studies that examined patients with stable angina pectoris, patients with myocardial infarction more than 3 months ago, or patients that had undergone coronary revascularization more than 6

months ago. The risk of having a recurrent event differs between the first months after a coronary event and the time period that follows. We tried to account for this by using the criterion of 3 months post-MI and 6 months post-procedure, which has previously been applied for such purposes (7, 8). Evidence exists that risk of recurrent events may be higher even up to a year after having unstable angina or an MI, while after this period the overall risk is reduced to the same level as that of stable CAD patients (6). To further account for such differences in risk, we only included studies that had follow-up durations longer than one year available. This enabled all patients to eventually reach a risk comparable to stable CAD patients. Nevertheless, the definition of stable CAD varied between the articles, as did the exclusion criteria, which influences the homogeneity and the comparability of the study populations.

CONCLUSION

Although multiple prediction models have been developed for adverse events in patients with established, stable CAD, the variables contained in these models display large heterogeneity. Differences in study design, study populations and incidence rates may in part account for this heterogeneity. Still, most models include classic risk factors such as age, diabetes, hypertension, smoking and cholesterol. The majority of existing studies have paid insufficient attention to model performance and validation.

Thus, development of a reliable prediction model for incident coronary events in patients with stable coronary disease is warranted. Such a model should preferably be based on a large, multinational population, use a clear and uniform definition of stable CAD, and should examine long-term follow-up that includes (cardiovascular) mortality as well as non-fatal cardiovascular adverse events. Performance assessment should be done including measures of discrimination, calibration, as well as overall performance measures. Moreover, attention should be paid to external validation.

Such a model is essential in order to serve as a starting-point to better prevent recurrent events in patients with stable coronary artery disease, and to herewith optimize secondary prevention strategies.

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2.2|

Development of prediction models for both fatal and non-fatal adverse events

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ABSTRACT

Background: Appropriate risk stratification of patients with established, stable coronary artery disease (CAD) may contribute to the prevention of recurrent cardiovascular events. The purpose of this study was to develop and validate risk prediction models for various cardiovascular endpoints in a large cohort of CAD patients.

Methods: the EUROPA database, consisting of 12 218 patients with established CAD, with a median follow-up of 4.1 years, was used. Cox proportional hazards models were used for model development. The endpoints examined were cardiovascular mortality, non cardiovascular mortality, non-fatal MI, coronary artery bypass grafting, percutaneous coronary intervention, resuscitated cardiac arrest, as well as combinations of these endpoints. Performance measures included Nagelkerke R^2, time-dependent ROC curves, and calibration-plots with the Hosmer and Lemeshow test.

Results: Backward selection resulted in a prediction model for cardiovascular mortality (646 events) that included age, current smoking, diabetes mellitus, total cholesterol, BMI, prior MI, history of congestive heart failure, peripheral vessel disease, prior revascularization, and prior stroke. Model performance was adequate for cardiovascular mortality with a Nagelkerke R^2 of 12%, and an AUC of 0.73. However, performance of models constructed for non-fatal and combined endpoints was considerably worse, with AUCs around 0.6.

Conclusions: In patients with established CAD, the risk of cardiovascular mortality during longer-term follow-up can be adequately predicted based on clinical characteristics which are available at baseline. However, the prediction of non-fatal outcomes, both separately and in combination with fatal outcomes, poses major challenges for clinicians and model developers.

INTRODUCTION

Since the late 1960s, coronary artery disease (CAD) mortality rate has declined (1, 2). Several developments have contributed to this decline. First, multiple aspects of patient risk profile have improved, which is partly due to preventive interventions (3, 4). Examples include a decline in serum total cholesterol concentration and prevalence of smoking. Moreover, improved treatment of stable CAD (statins, beta blockers, and ACE-inhibitors) and acute coronary syndromes, including myocardial infarction (MI), has been enabled by the introduction of reperfusion therapy with thrombolysis or PCI with stent implantation and the addition of antiplatelet agents to aspirin (5-7)**.** Also, improved cardiac rehabilitation after MI or CABG has resulted in a reduction of the total number of re-hospitalizations, although the results were modest (8-11). Nevertheless, CAD is still the leading cause of death in the Western world (12). It has been estimated that by the year 2020, nearly 20.5 million deaths worldwide will be due to cardiovascular disease. (13)

This trend may be altered by installing further preventive measures. In order to do this most effectively, first, appropriate risk stratification tools should be developed. Focusing on patients with established CAD for prevention of recurrent events and mortality may contribute to efficient healthcare, because these patients are at high risk of needing medical attention and may benefit most from additional treatment. Appropriate risk stratification in this population may identify patients that could be followed-up more closely, may be treated more aggressively, and whose compliance to prescribed drugs may be monitored more carefully, in order to prevent recurrent events.

While several risk stratification models have been designed for primary prevention, such as the Framingham risk score, the SCORE project, Prospective Cardiovascular Münster Study (PROCAM) and QRISK, (14-17) risk stratification models for patients with established CAD are less abundant (18-22) and no consensus currently exists on which is most appropriate. Furthermore, existing models (18-21, 23-25) have several limitations. These include retrospective study design (19), different endpoints, lack of validation (20), lack of uniformity in baseline characteristics because of a long inclusion period during which changes in treatment recommendations have occurred (21), and focus on specific ethnic groups (25).

In the current study, we set out to develop and validate a series of risk prediction models for different endpoints in a prospective cohort of European patients with established CAD. Our cohort consists of over 12 000 patients, making this the largest study to date to develop such a model. Endpoints examined included cardiovascular mortality, non cardiovascular mortality, non-fatal MI, coronary artery bypass grafting (CABG), percutaneous coronary intervention (PCI), and resuscitated cardiac arrest, as well as combinations of these endpoints.

METHODS

Study design, patients and data collection

The design of the EUROPA study has been reported elsewhere (26). In brief, this randomized, double-blind, placebo-controlled trial investigated the efficacy of perindopril in reduction of cardiovascular events in 12 218 patients. Informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the Declaration of Helsinki.

Study participants consisted of men and women 18 years or older, with evidence of coronary heart disease documented by previous MI (>3 months before screening), percutaneous or surgical coronary revascularization (> 6 months before screening), angiographic evidence of at least 70% narrowing of at least one major coronary artery, or (in men) a history of typical chest pain with an abnormal stress test. Informed consent was obtained from all patients. Exclusion criteria included clinically evident (NYHA ≥ 2) heart failure, planned revascularization procedure, hypotension (sitting systolic blood pressure <110 mmHg), uncontrolled hypertension (systolic blood pressure >180 mmHg and/or diastolic blood pressure >100 mmHg), use of ACE-inhibitors or angiotensin-2 receptor blockers in the last month, renal insufficiency (serum creatinine >150 µmol/L or 1.5 mg/dL), and serum potassium >5.5 mmol/L. Patients were randomly assigned to perindopril 8 mg or placebo once daily for at least 3 years. The first patient was enrolled in October 1997.

At baseline, exposure data were collected for age, current smoking (patients who were current smokers or smoked in the previous month), diastolic blood pressure (mmHg), systolic blood pressure (mmHg), heart rate (beats/min), diabetes (known history of diabetes or use of antidiabetic agents), total cholesterol (mmol/L), BMI (kg/m^2), family history of coronary artery disease, history of congestive heart failure, history of peripheral vessel disease, history of prior MI, history of prior revascularization, and prior stroke.

Patients were followed up for cardiovascular mortality, non cardiovascular mortality, myocardial infarction, CABG, PCI, and resuscitated cardiac arrest until March 2003. Intensive monitoring and endpoint validation was done by a Clinical Event Committee (CEC) (27). Median follow-up was 4.1 years (interquartile range 4.0–4.5 years).

STATISTICAL METHODS

Development of the risk prediction models

Several techniques are available for the development of risk prediction models with subsequent internal validation, including the split-sample and bootstrap methods. According to the split-sample method, the original sample is (randomly) divided into two parts: a training set for model development and a testing set for model validation (28).

Bootstrap replicates the process of sample generation from an underlying population by drawing 'bootstrap samples' with replacement from the original sample; the bootstrap samples usually have the same size as the original (29).

Table 1. Patient baseline characteristics stratified by training set and testing set*

	Training set (n=8144)	Testing set (n=4074)	p-value
Age, years (SD)	60 (9.30)	60 (9.38)	0.89
Male	6965 (85.5)	3474 (85.3)	0.71
Current smoking	1250 (15)	612 (15)	0.64
Diastolic blood pressure (mmHg), mean (SD)	82 (8)	82(8)	0.50
Systolic blood pressure (mmHg), mean (SD)	137 (16)	137 (15)	0.81
Total cholesterol (mmol/L), mean (SD)	5.37 (1.05)	5.36 (1.05)	0.66
Diabetes	1021 (12.5)	481 (11.8)	0.25
BMI (kg/m^2), mean (SD)	27.4 (3.5)	27.4 (3.5)	0.72
eGFR ($mL * min^{-1} * 1.73\ m^{-2}$), mean (SD)	75 (20)	75 (20)	0.71
Heart rate (bpm), mean (SD)	68 (10)	68 (10)	0.18
Peripheral vessel disease	581 (7.1)	302 (7.4)	0.58
Family history of coronary artery disease	2173 (26.7)	1155 (28.4)	0.05
Congestive heart failure	105 (1.3)	48 (1.2)	0.60
Prior MI	5267 (64.7)	2643 (64.9)	0.81
Prior revascularization	4454 (54.7)	2255 (55.4)	0.49
Prior stroke	154 (1.9)	68 (1.7)	0.39

* Results are presented as (n) % unless otherwise indicated

According to the bootstrap method, risk prediction models are developed on the original sample and validated in the set of bootstrap samples. The bootstrap method is preferred in small datasets. Our original sample was large, so that we were confident to apply the split-sample method: the original sample was randomly divided into a training set of 8144 patients (two thirds of the original sample) and a testing set of 4074 patients.

To develop the optimal risk prediction model, multivariable Cox proportional hazards analysis was applied in the training set. All available predictors, as described under 'study design', were considered as potential determinants of the outcomes that we studied. Backward stepwise selection was used for variable selection, because this has been argued to render reliable predictors.(28) Variable exclusion was performed using a 5% significance level as a stopping criterion. We elected not to apply the Lasso-shrinkage method for variable selection [36], since this method extensively uses computer CPU time, particularly so in large datasets, whereas it has no major advantages over stepwise selection in such large datasets.

We examined the endpoints cardiovascular mortality, non cardiovascular mortality, non fatal MI, CABG, and PCI. Moreover, we examined the combination of cardiovascular mortality, non-fatal MI, and resuscitated cardiac arrest, which was originally the primary endpoint of the EUROPA study (combined endpoint 1(22)), and the combination of cardiovascular mortality, non cardiovascular mortality, non fatal MI, CABG, PCI, and resuscitated cardiac arrest (combined endpoint 2). In the analysis of the combined endpoints, we applied censoring at the first moment that any one of the endpoint components occurred in a patient. In the analysis of the separate outcomes, we additionally accounted for competing risk, by using time-dependent covariates. For instance, when CABG was the endpoint of interest, we used non-fatal MI and PCI as time-dependent variables.
Complete information was available on most variables. Values for total cholesterol, heart rate, history of MI and revascularization, and BMI were missing in less than 5% of participants. Missing values were handled using expectation maximization(30), which is an iterative method for finding maximum likelihood estimates of parameters in statistical models, where the model depends on unobserved latent variables. SPSS 17.0 for Windows was used for the above-described analyses.

Validation of the risk prediction models

After deriving the models in the training set, we assessed their performance in the testing set. We used Nagelkerke's R^2 to assess global model performance (28). R^2 is a likelihood-based measure that provides information about the goodness of fit of the model, or in other words, how well the regression line estimates the real survival. There are several different definitions of R^2 (28). The definition proposed by Nagelkerke can readily be applied to survival outcomes has the advantage of being scaled between 1 and 100%. Of note is that the value of R^2 partly depends on incidence of the outcome. Lower incidence results in lower values of R^2, which thus should be interpreted in their appropriate context (31) Subsequently, we assessed model discrimination for every endpoint by calculating area under the receiver operating characteristic curve (AUC). Model discrimination is the ability of the model to rank persons appropriately, from low to high risk. We calculated time-dependent AUCs using the statistical program R (32). This approach takes into account the follow-up time until event occurrence. Standard errors (SEs) were calculated by bootstrapping.
To assess differences in model discrimination between the testing set and the validation set, we compared the AUCs with the method of Hanley and McNeil (33) by using MedCalc (34). A two-tailed probability < 0.05 was considered a statistically significant result.
Moreover, we investigated calibration, or how closely the predicted probabilities reflect actual risk. For this purpose, we compared observed survival, derived from Kaplan Meier curves, with predicted survival, calculated from the Cox proportional hazards models. We

constructed calibration plots based on categories defined by deciles of predicted risk. To test the goodness-of-fit we performed the Hosmer-and-Lemeshow test.

RESULTS

Baseline characteristics are summarized in Table 1. Mean age was 60 years and 85% were men. No significant differences were present between the training and testing sets. Incidence of the endpoints is displayed in table 2.
Incidence of cardiovascular mortality was 9.6 per 1000 person years in the training set.

Table 2. Event rates of the endpoint in the training set and the testing set.

Outcome	Total number of events	Event rate training set (per 1000 person years)	Event rate testing set (per 1000 person years)
Cardiovascular mortality	464	9.6	8.1
Non cardiovascular mortality	323	6.1	6.8
Non-fatal MI*	673	13.6	12.4
PCI **	671	13.4	12.7
CABG[§]	564	11.6	10.0
Combined endpoint 1[#]	1091	22.9	20.2
Combined endpoint 2[§§]	2188	43.6	47.1

*MI = myocardial infarction; ** PCI= Percutaneous Coronary Intervention, [§]CABG =coronary artery bypass grafting; [#] combined endpoint 1 = cardiovascular mortality + non-fatal MI + resuscitated cardiac arrest,; [§§]combined endpoint 2= cardiovascular mortality + non cardiovascular mortality + MI + CABG + PCI + resuscitated cardiac arrest

Model development

In the training set, 16 potential variables were evaluated for model inclusion. The variables included in the best-fitting prediction model for cardiovascular mortality after backward selection were age, current smoking, diabetes mellitus, total cholesterol, BMI, prior MI, history of congestive heart failure, peripheral vessel disease, prior revascularization, and prior stroke (table 3). Hazard ratios for the variables in this model are displayed in table 4. A smaller number of variables were included in the predictive model for non cardiovascular mortality after backward selection, namely age, current smoking, and heart rate. Variables included in the prediction models for MI, CABG, PCI and the combined endpoints are also displayed in table 3.

Table 3. Prognostic models resulting from backward stepwise selection, with corresponding area under the ROC curve and Nagelkerke R^2 in the training set and testing set.

Endpoint	Full model resulting from backward stepwise selection	training set		testing set		P-value for difference in AUC between training and testing set
		*AUC (95% confidence interval)	Nagelkerke R^2	*AUC (95% confidence interval)	Nagelkerke R^2	
Cardiovascular mortality	age, current smoking, diabetes mellitus, total cholesterol, BMI, prior MI, history of congestive heart failure, peripheral vessel disease, prior revascularization, and prior stroke	0.70 (0.69-0.71)	10%	0.73 (0.70-0.77)	12%	0.16
Non cardiovascular mortality	age, current smoking, and heart rate	0.69 (0.67-0.71)	5%	0.71 (0.69-0.73)	8%	0.22
Non-fatal MI[%]	age, current smoking, diabetes mellitus, total cholesterol, prior MI, family history of CAD, and peripheral vessel disease	0.60 (0.58-0.62)	2%	0.59 (0.56-0.62)	2%	0.48
CABG**	age, gender, diabetes mellitus, total cholesterol, BMI, prior MI, family history of CAD, and prior revascularization	0.67 (0.64-0.68)	5%	0.65 (0.62-0.68)	4%	0.28
PCI[§]	diabetes mellitus, total cholesterol, and prior revascularization	0.55 (0.54-0.56)	1%	0.56 (0.53-0.58)	1%	0.43
Combined endpoint 1[#]	age, gender, current smoking, diabetes mellitus, total cholesterol, diastolic blood pressure, renal function, prior MI, peripheral vessel disease, prior revascularization, and prior stroke	0.64 (0.63-0.66)	5%	0.63 (0.61-0.66)	6%	0.45
Combined endpoint 2[§§]	age, gender, current smoking, diabetes mellitus, total cholesterol, family history of CAD, peripheral vessel disease, prior revascularization, and prior stroke	0.62 (0.60-0.63)	4%	0.61 (0.59-0.63)	4%	1.0

* AUC = area under the ROC curve, **CABG = Coronary Artery Bypass Grafting, [%]MI = myocardial infarction, [§]PCI= Percutaneous Coronary Intervention, [#]combined endpoint 1 = cardiovascular mortality + non-fatal MI + resuscitated cardiac arrest, [§§]Combined endpoint 2 = cardiovascular mortality + non cardiovascular mortality + MI + CABG + PCI + resuscitated cardiac arrest

Model performance in the training set

Nagelkerke's R^2 and time-dependent AUC were calculated for each endpoint (table 3). Overall performance, assessed with Nagelkerke R^2, was 10% for cardiovascular mortality. Overall performance was worse for the other outcomes. The model for cardiovascular mortality risk prediction displayed the best discrimination with an AUC of 0.70. The prediction models for non cardiovascular mortality, MI, CABG, PCI and the combined endpoints 1 and 2 resulted in lower AUCs of 0.69, 0.60, 0.67, 0.55, 0.64 and 0.62 respectively. Model calibration, as assessed by the Hosmer and Lemeshow test, was adequate. The Chi square values and corresponding p-values for all endpoints are displayed in table 5.

Table 4. Hazard ratios for cardiovascular mortality for baseline variables included in the prediction model.

Variable	Hazard ratio (95% confidence interval)	P-value
Age (years)	1.06 (1.04-1.07)	< 0.001
Current smoking	1.96 (1.47-2.62)	< 0.001
Diabetes Mellitus	1.01 (0.76-1.35)	0.93
Total cholesterol (mmol/L)	1.25 (1.12-1.39)	< 0.001
BMI*	1.01 (0.98-1.05)	0.39
Prior MI**	1.96 (1.48-2.58)	< 0.001
History of congestive heart failure	3.10 (1.84-5.23)	< 0.001
Peripheral vessel disease	1.43 (1.04-1.98)	0.03
Prior revascularization	0.98 (0.77-1.23)	0.83
Prior stroke	1.77 (1.09-2.88)	0.02

*BMI = Body Mass Index; **MI = Myocardial Infarction

Model performance in the testing set

The models were subsequently validated in the testing set. Values for Nagelkerke R^2 indicated a good overall performance for cardiovascular (12%) and non cardiovascular mortality (8%) (table 3). For the other outcomes, overall performance was worse, and values were in a similar range as those in the training set. Discrimination was comparable to the training set (including an AUC=0.73 for cardiovascular mortality). The mean and confidence intervals of the AUCs for both sets are also displayed in table 3. AUCs were not significantly different between the training and testing set.

Figure 1 a., 1 b., 1 c., and 1 d. show the calibration plots for cardiovascular mortality, non-fatal MI and combined endpoints 1 and 2 in the testing set. Because of the relatively low event rate in this study population (9.6/1000 person years for cardiovascular mortality and 13.6/1000 person years for non-fatal MI), the observed fraction of the population that remained event-free ranged between 0.8 and 1.0 for each decile of predicted probability

of being free from cardiovascular mortality. These deciles of predicted probability of being event-free also ranged from 0.7 upwards. As such, the calibration plot only displayed values in the upper range of both the x and y-axis. A similar situation occurred during evaluation of the prediction model for non-fatal MI and the models for the combined endpoints. It was not possible to construct calibration plots for non cardiovascular mortality, PCI and CABG, because survival was even closer to 1.0 for these endpoints. As such, these calibration plots were not informative. Nevertheless, calibration as evaluated by the Hosmer and Lemeshow tests was sufficient (table 5).

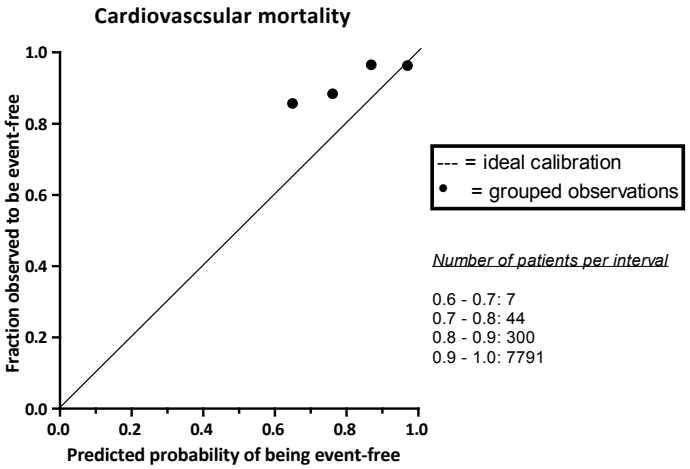

Figure 1a. calibration plot cardiovascular mortality, four years follow-up

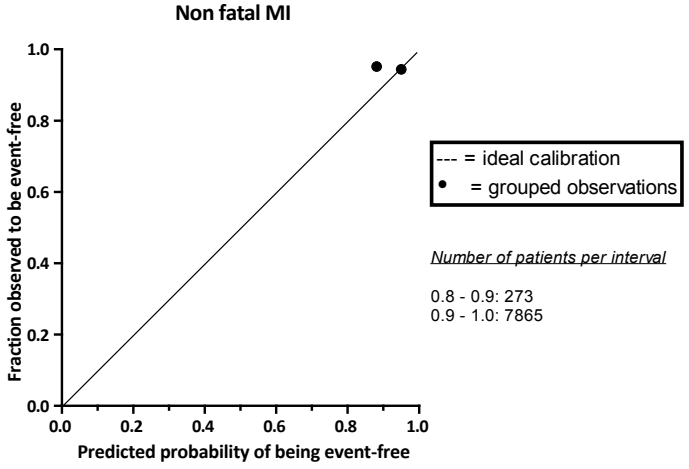

Figure 1b. calibration plot non fatal MI, four years follow-up

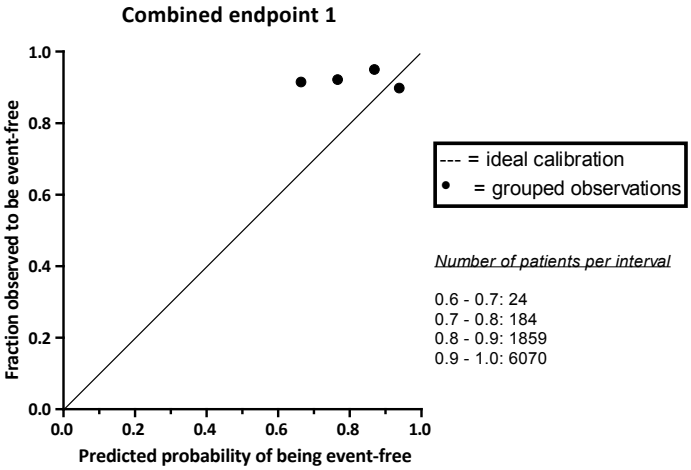

Figure 1c. calibration plot combined endpoint 1, four years follow-up

*combined endpoint 1 = cardiovascular mortality + non-fatal MI + resuscitated cardiac arrest;

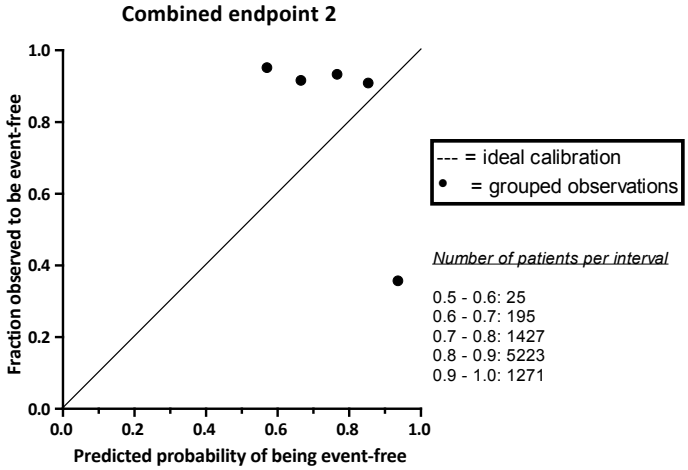

Figure 1d. calibration plot combined endpoint 2, four years follow-up

§§combined endpoint 2 = cardiovascular mortality + non cardiovascular mortality + non-fatal MI + CABG + PCI + resuscitated cardiac arrest

Table 5. Model calibration as assessed by the Hosmer and Lemeshow test

	Training set		Testing set	
	Chi square	**p-value**	**Chi square**	**p-value**
Cardiovascular mortality	3.61	0.89	13.77	0.09
Non cardiovascular mortality	6.84	0.55	3.50	0.90
Non-fatal MI*	5.84	0.67	4.26	0.83
PCI **	14.80	0.06	2.23	0.97
CABG[§]	3.97	0.86	9.83	0.28
Combined endpoint 1[#]	20.43	0.01	16.56	0.04
Combined endpoint 2[§§]	1.98	0.98	8.58	0.38

*MI = myocardial infarction; ** PCI= Percutaneous Coronary Intervention, [§]CABG =coronary artery bypass grafting; [#]combined endpoint 1 = cardiovascular mortality + non-fatal MI + resuscitated cardiac arrest; [§§]combined endpoint 2 = cardiovascular mortality + non cardiovascular mortality + non-fatal MI + CABG + PCI + resuscitated cardiac arrest

DISCUSSION

Risk prediction models and ensuing risk scores may be used in CAD patients to adjust individual treatment to the patient's risk profile (18). Furthermore, they may provide patients with further insight into their individual risk and thus motivate them to reduce modifiable risk factors (35). In this paper, we developed and validated a comprehensive set of prediction models in patients with established, stable coronary artery disease, to estimate the risk of cardiovascular mortality, non cardiovascular mortality, non-fatal MI, CABG or PCI. The model for the prediction of cardiovascular mortality, which is the 'hardest' endpoint, displayed good overall performance with Nagelkerke's R^2 being 12%,. Furthermore, the model displayed good discrimination, with an AUC of 0.73. The model was well-calibrated, as assessed by the Hosmer and Lemeshow test.

Our main results show that cardiovascular mortality in CAD patients can to a large extent be predicted by 'established' clinical risk factors such as DM and cholesterol level, and by history of vascular disease including CAD and peripheral vessel disease. These traditional risk factors are easily obtainable during patient assessment. Of note is that, while blood pressure was part of the prediction model for the combined endpoint, it did not predict cardiovascular mortality in our data. These seemingly discrepant findings may partly result from the fact that a large part of the study population was using ACE inhibitors and beta blockers. Established risk factors were also implicated in the INTERHEART study, a case-control study performed in 52 countries worldwide, which examined primary prevention, and demonstrated that traditional risk factors account for 90% of the population attributable risk of MI (36). Earlier studies by St. John Sutton et al. (37) and Maas et al. (38), that studied long-term survival after MI in more than 500 patients, also implicated

established risk factors. These studies included demographic variables, co-morbid conditions as well as procedural variables in their models and showed that age (37, 38), history of prior MI (37, 38), congestive heart failure (37), left ventricular function (37, 38), and multivessel disease (38) were independent predictors of long-term mortality. Deckers et al (22) previously examined risk factors for cardiovascular events within the EUROPA study, using a combined endpoint. In the present paper, we examine additional outcomes and further explore aspects of model performance.

Although, as mentioned above, several studies have examined the associations of risk factors with recurrent events, the number of risk prediction models that have been developed for patients with established CAD is limited. Examples of prediction models that have previously been developed and validated include those of Clayton et al. (18), Prugger et al. (19), Singh et al.(21) and Blankenberg et al.(23). The demographic variables and co-morbid conditions they used in their models largely concur with our variables, including age, smoking, prior MI, and history of congestive heart failure. In addition, Sing et al. (21) included several procedural variables obtained from index PCI, while Blankenberg et al. (23) added biomarkers to inventorise whether this improves model performance. They calculated AUCs of 0.74 and 0.69, respectively, using combined endpoints which included all-cause mortality and MI, and MI, stroke, and cardiovascular mortality, respectively. While the performance of our models for the prediction of cardiovascular and non cardiovascular mortality is comparable to that of these models, we also demonstrate that model performance is considerably worse for non-fatal MI, PCI and CABG examined as separate outcomes, which has not been examined previously.

Previous existing models for cardiovascular risk prediction in CAD patients have several limitations (18-21, 23-25) Clayton et al. (18) developed a risk score in patients with stable symptomatic angina and examined occurrence of all-cause mortality, MI or disabling stroke, but did not assess model performance. The same was true for Marschner et al. (24). They developed a multivariate risk factor model in 9014 patients with acute MI or hospitalization for unstable angina, but performance was not assessed. Prugger et al. examined the relationships between cardiovascular risk factors and long-term mortality in CAD patients, however, they used a retrospective study design. Singh et al. (21) designed a model in 9165 patients undergoing PCI, but their study lacks of uniformity in baseline characteristics because of a long inclusion period (7 years) during which changes in treatment recommendations have occurred. Marchioli et al. (25) included over 11000 Mediterranean patients to develop a risk chart. However, they included patients directly after occurrence of MI. Such patients are known to have higher risk of recurrent events in the first months after their index event, which complicates model development. Furthermore, this was a specific group (Mediterranean patients), and therefore generalizability of the chart is limited. A comprehensive review of the strengths and limitations of CAD policy

models in CAD patients developed so far is given by Unal et al. (20) Likewise, they noted that the biggest part of the models were either not calibrated or not validated.

Most studies that have developed prediction models for CAD patients did not include PCIs and CABGs as outcomes, nor did they examine these outcomes separately. To provide an extensive framework for cardiovascular disease prediction, we chose to also examine these outcomes in the current study. When we examined non-fatal MI, CABG and PCI separately, model performance and discrimination were notably lower than those for cardiovascular mortality. For non-fatal MI, this may in part be explained by the fact that this endpoint is not a pathophysiological entity, as part of the patients that experience MI die immediately. Moreover, there is a difference between acute risk, which is most often related to thrombosis, and long-term risk, which is related to the evolution of the atherosclerotic process. Hence, we also investigated a combined endpoint containing non-fatal MI, as well as cardiovascular mortality and resuscitated cardiac arrest. Nevertheless, discrimination remained relatively low for this combined endpoint with a c-statistic of 0.64. For PCI and CABG, worse model performance and discrimination may in part be explained by the fact that undergoing revascularization is heavily influenced by the opinion of the treating physician (39).

The calibration-plots of the prediction models for non-fatal endpoints were not informative, because of the relatively low incidence rates of the separate endpoints. We also examined two combined endpoints that included fatal as well as non-fatal events. We found that the relatively poor model performance for non-fatal outcomes influenced model performance for both combined endpoints as well. Addition of non-fatal MI and resuscitated cardiac arrest to the endpoint cardiovascular mortality (resulting in combined endpoint 1) resulted in a reduction of the c-statistic from 0.7 to 0.64. Concerning combined endpoint 2, since non-fatal MI, CABG, PCI and resuscitated cardiac arrest were the first events to occur in 66.8% of subjects that suffered any event, the AUC of this combined endpoint was closer to the AUC of these non-fatal endpoints, namely 0.62. Furthermore, the calibration-plot for combined endpoint 2 showed discrepancies in the highest decile of predicted probability of being event-free. This was partly due to the fact that follow-up time until the occurrence of non-fatal events was generally shorter than that until the occurrence of fatal events. Furthermore, the number of non-fatal events was much higher than the number of fatal events. These results suggest that when modeling prognosis in patients with stable CAD, study endpoints should be chosen with care, as pooling several outcomes may influence model performance to a great extent.

Strengths of our study include the availability of a large, prospective CAD patient cohort for model development, and the availability of a wide range of risk factors, whose importance we were able to explore for the prediction of non-fatal as well as fatal cardiovascular outcomes. Moreover, since our study consists of a multicenter, multinational population, it

is likely that the results apply to a broad range of clinical practices. Heterogeneity in center-specific policies was minimized by a uniform study protocol. Furthermore, we performed an extensive assessment of model performance, which is key for model appraisal (20). We applied multiple methods to do so, which has been recommended (40). Notably, we used time-dependent receiver operating characteristic curves (ROC), thus taking follow-up time into account, in contrast with previous studies (21, 23).

Several aspects of this study warrant further consideration. Although the absolute number of events was high (combined endpoint 2, n=2188), incidence rate was relatively low, which posed challenges for constructing informative calibration-plots for the non-fatal endpoints. Furthermore, follow-up was limited to four years, which hampers longer-term prediction. Also, the EUROPA study is a clinical trial with strict in- and exclusion criteria, which resulted in a very specific study population. Moreover, we did not have biomarker information available, which may have further improved model performance. However, although studies investigating incremental value of biomarkers over 'established' cardiovascular risk factors have rendered significant results, absolute differences in model performance, as assessed by measures such as the AUC, have been modest so far.

Various performance measures may be applied in prognostic modeling (28) We strived to provide a comprehensive assessment of model performance by applying measures of overall performance as well as measures of model discrimination and calibration. To assess overall model performance, we used Nagelkerke's R^2. The value of R^2 partly depends on the prevalence of the outcome variable in the dataset (40). Thus, occasionally, if event rates are low in survival data, R^2 may be relatively low, and should be interpreted within its appropriate context (40). This also holds for the current study; event rates were relatively low because we examined patients with stable CAD. Furthermore, while the AUC increases linearly as the fraction of cases identified as high-risk by the model decreases, R^2 increases hyperbolically (40). This may also lead to relatively low R^2 values for models that may be considered to have good overall performance.

CONCLUSIONS

The models we have developed in the current study illustrate the potential of established cardiovascular risk factors to serve as risk stratification tools for CAD patients. The prediction models for cardiovascular and non cardiovascular mortality performed well with time-dependent AUCs of 0.73 and 0.72, respectively, and Nagelkerke's R^2 of 13% and 8%, respectively. Performance of prediction models for non-fatal MI, CABG and PCI in the same dataset was considerably worse, and thus prediction of these outcomes poses major challenges for clinicians and model developers. Caution should be used when examining these types of clinical outcomes or combined endpoints that include these outcomes.

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3

Improving the state-of-the-art: dynamic prediction models

13

Improving the state-of-the-art: dynamic prediction models

3.1

Principles of micro-simulation modeling

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ABSTRACT

Background: In cardiovascular disease, numerous evidence-based prognostic models have been created, usually based on regression analyses of isolated patient datasets. They tend to focus on one outcome event, based on just one baseline evaluation of the patient, and fail to take the disease process in its dynamic nature into account. We present so-called micro-simulation as an attractive alternative for clinical decision-making in individual patients. We aim to further familiarize clinicians with the concept of micro-simulation and to inform them about the modelling process.

Methods and results: We describe the modelling process, advantages and disadvantages of micro-simulation. We illustrate the concept using a hypothetical 60 year old patient, with several cardiac risk factors, who is hospitalized for myocardial infarction. By using micro-simulation, we calculate this patient's probability of death. In our example, this particular patient's estimated life-expectancy turns out to be 8.9 years. While calculating this life-expectancy, we were able to account for multiple outcome events and changing patient characteristics.

Conclusions: Micro-simulation takes into account the dynamic nature of coronary artery disease by estimating most likely outcomes regarding a broad range of clinical events. Moreover, micro-simulation can be used to evaluate treatment effects by estimating the event-free life expectancy with and without treatment. Hence, micro-simulation has several advantages compared to modelling techniques such as regression.

INTRODUCTION

Predictive thinking plays a fundamental role in medicine. Before certain therapy is initiated, a physician must consider the probability that the patient will improve or deteriorate without such therapy, the chances of improvement if the therapy is initiated, the risks of adverse events, and the therapy-related costs.[1] The improvement of our predictive ability is one of the driving forces behind clinical research.[2, 3]

Numerous evidence-based prognostic models have been created to help physicians make optimal and consistent decisions, usually based on regression analyses of isolated patient datasets.[4, 5] These so-called parametric models tend to focus on one outcome event, and are based on one baseline evaluation of the patient, thus failing to take the disease process in its dynamic nature into account. An alternative for clinical decision-making in individual patients is micro-simulation.[6] Micro-simulation replicates individual patient histories, and may thus inform physicians by estimating the most likely outcomes regarding a broad range of clinical events.

In this paper, we will present micro-simulation as an attractive alternative for parametric models for clinical decision-making in individual patients. While the general technique of micro-simulation is applicable to various disease areas, it has not yet been applied in coronary artery disease (CAD). During the last decades, our understanding of CAD has considerably improved, and major progress has been achieved in patient management and outcome.[7] Although CAD is a common disease area,[8] the number of prediction models that has been developed for patients with established CAD is limited.[9, 10]

The aim of this paper is to further familiarize clinicians with the concept of micro-simulation and to inform them about the modelling process, which is often perceived as a 'black box'.

CLASSICAL CONCEPTS

Regression models

Newly developed therapies are usually introduced in clinical practice after proof of their efficacy and safety by clinical trials. The results of these trials apply by necessity to a patient population that is defined by the trial eligibility criteria. In clinical practice, however, physicians don't treat populations, but individual patients, in whom the benefits, risks, effectiveness, and cost-effectiveness of therapy depend on a large number of characteristics. To provide physicians with tools for rational clinical decision making, cardiovascular risk models have been created for several categories of patients with (suspected) atherosclerotic disease, which are based on the results of regression analyses that relate patient characteristics to treatment effects and outcome.[4, 11, 12] Logistic regression and Cox proportional hazard regression are most frequently used for this purpose.

Meet Mr. Jones, a 60-year old accountant without cardiac history. He is admitted to the coronary care unit with severe chest pain, which originated 4 hours ago, and significant ST-segment elevations in V3-V5. Mr. Jones has no signs of heart failure, and is hemodynamically stable with a pulse of 70 b/m and a blood pressure of 130/80 mmHg. What is his estimated probability of death during hospitalisation?

Both logistic regression and Cox proportional hazard regression may provide the answer. These regression methods provide a mathematical formula that relates a set of explanatory variables to the probable occurrence of one binary outcome event, based on one baseline evaluation of the patient.[13] Logistic regression is appropriate if follow-up duration is limited and incidence of the outcome event is infrequent. Otherwise, Cox' proportional hazards regression is recommended.[14] Typical examples of logistic regression models are the TIMI and GUSTO-1 risk scores for the prediction of 30-day mortality after admission with ST-elevation myocardial infarction[4, 5], and the EuroSCORE for the prediction of hospital mortality in adult patients undergoing cardiac surgery[15]. According to the TIMI and GUSTO-1 risk scores, Mr. Jones's 30-day mortality probability is approximately 2-3%. Cox regression was applied to develop the SCORE chart to estimate the 10-year risk of fatal cardiovascular events in individuals in the general European population.[16]
Especially in situations that require immediate decisions, the simplicity of the methods has stimulated the application of regression models and the practice of evidence-based medicine.[17] The main disadvantage of regression models is that they typically describe only one outcome event, based on one baseline patient evaluation. Herewith, they fail to take into account the dynamic nature of the disease process. When the objective is to provide predictions over an extensive time-span, by using for example a traditional Cox analysis, the probability that baseline characteristics will have changed over time is large, which influences the accuracy of the predicted prognosis.
This may be in part accounted for when repeated measurements are available. These may be incorporated into the Cox model as risk factors whose value changes over time. Basically, in such a "time-dependent" analysis, the complete follow-up time for each patient is divided into different time windows and for each time-window, a separate Cox analysis is carried out using the specific value of the time-dependent variable.[18] However, the maximum number of consecutive Cox analyses that may thus be combined is limited for computational reasons.[18] Therefore, if the value of a time-dependent risk factor changes several times, this approach poses challenges. Hence, it is only suitable to address relatively short-term effects[18].

Cardiovascular risk as a dynamic phenomenon

The management of patients that have experienced a coronary event is a dynamic process. In fact, throughout hospitalisation a continued refinement of the initial treatment strategy is necessary to treat the patient as well as possible. For example, Mr. Jones will be treated with a percutaneous coronary intervention (PCI) directly when he arrives to the hospital. To prevent recurrent events he will receive cardiac medication including anticoagulants. As such, his 'baseline characteristics' change: he now has a cardiac history, consisting of myocardial infarction and primary PCI. Suppose that Mr. Jones initially survives the coronary event and the PCI, but experiences a hemorrhagic stroke on day 3: then the treatment strategy and risk of death during his hospitalisation will have changed. Therefore, it would be logical to update risk assessments continuously, based on actual patient status and intervening events. For Mr. Jones this means that 'previous stroke' will be added to his profile and the use of anticoagulants will be removed. His risk for recurrent events will again be different now.

The concept of dynamic prognostication has been introduced for patients presenting with non-ST-elevation acute coronary syndromes.[19] Based on the GUSTO-IIb and PURSUIT databases, several logistic regression equations were developed to predict 30-day mortality using baseline information, and variables obtained during hospitalisation. These separate regression models were then integrated into a composite, dynamic model to describe the effects of changing conditions over time. The performance of this model was considerably better than the 'static' regression model that used only baseline characteristics.[19]

The approach of dynamic risk modelling may also be applied in other settings such as established, stable CAD.[20] In general, this approach may provide a valuable guidance for the short-term management of patients with established CAD; however, as far as long-term patient management is concerned, it becomes rather impractical. More importantly, such dynamic risk decision-models remain focused on one outcome event, do not provide detailed insight into the life history of a patient, and cannot take into account the versatile nature of atherosclerotic disease because of the limited follow-up time. In order to overcome these limitations the clinical course of a patient with CAD can be described by a simulation model.

SIMULATION MODELLING

Simulation modelling refers to the process of imitating a real-life phenomenon with a set of mathematical equations, which are based on observational data, using computer software[21]. In medicine, simulation models can be used to study the natural course of a disease, as well as the effects of chronic treatment,[22] and can apply for populations or individual patients. We will describe the Markov state transition model and micro-simulation.

Markov state transition model

The application of Markov models for determining prognosis has been first described in 1983.[23]-[24] According to this modelling technique, individuals from a virtual patient population are distributed across distinct health states (so-called Markov states) and subsequently make transitions between these states.[23] In order to simulate their remaining course of life, the virtual time-axis is divided into a finite number of time intervals whose length can vary according to the probability of making a transition between states (i.e. having an event).[25] At the end of each interval, patients who are still alive can move to a different state according to predefined transition probabilities. Consequently, the time spent in a certain state depends on the incidence of the predefined events. Rare events result in a longer time spent in a certain state than common events.

The transitions between states continue until the entire cohort is absorbed into the 'dead' state or until the cycle sum becomes too low, and subsequently the simulation ends.[23] The output usually includes the estimated population average life expectancy. Treatment effects can be studied by repeating the simulation with adjusted probabilities.

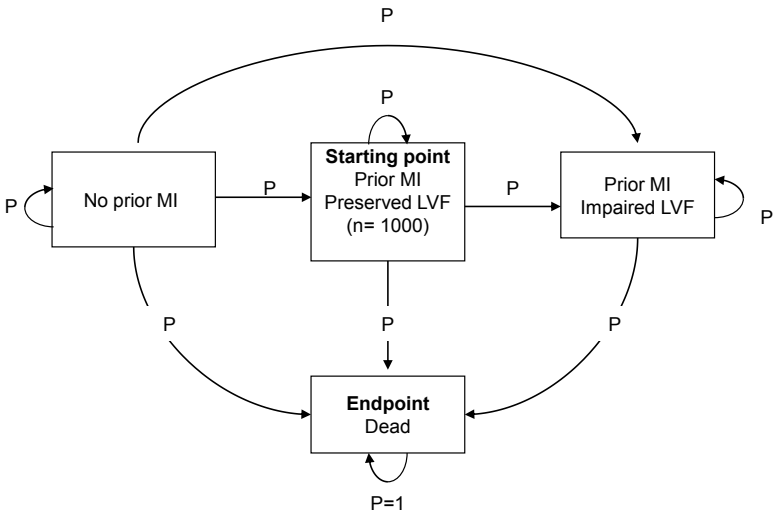

Figure 1. Simple Markov model to study the lifetime clinical course of CAD patients

Mr. Jones has experienced his first MI, and thus has established CAD now. He falls into the category of "prior MI, preserved left ventricular function (LVF)". The simple Markov model that is presented in Figure 1 and panel A and B in table 1, might be used to study the virtual lifetime of a cohort of 1000 patients who are copies of Mr. Jones. In this example, the probabilities of changing health state are independent of patient characteristics and are based on probabilities obtained from prior research. At the beginning of the first model

cycle, all patients are in the health state 'Prior MI, preserved LVF' (Panel B, row: 1, column: 'prior MI, preserved LVF'; this cell contains 1000 patients). Panel A shows fictive transition probabilities between the different health states. As can be seen in panel A, row 2, the probabilities of staying in the health state 'Prior MI, preserved LVF', or moving from this state to the states 'prior MI, impaired LVF' or 'Dead', are 0.85, 0.05 and 0.10, respectively. Hence, at the end of the first cycle (i.e. at the end of the first year), 850 patients will have remained in this state (1000 patients multiplied by the transition probability of 0.85 from panel B), and 50 patients will have moved to the health state 'Prior MI, impaired LVF' (1000 patients multiplied by the transition probability of 0.05). These patients are still alive, but their prognosis has changed. Another 100 patients will have moved to the health state 'dead' (1000 patients multiplied by 0.10). This process is repeated for the second year of follow-up, again using the appropriate transition probabilities from panel A.

Table 1. Panel A Input and output of a simple Markov model to study the lifetime course of 1000 patients of age 60 without prior myocardial infarction

Health state before transition	Health state after transition			
	Prior MI	Prior MI	Prior MI	Dead
		Preserved LVF	Impaired LVF	
No prior MI	0.94	0.04	0.005	0.015
Prior MI – preserved LVF	0	0.85	0.05	0.10
Prior MI – impaired LVF	0	0	0.85	0.15
Dead	0	0	0	1

Input: transition probabilities (related to a cycle-interval length of 1 year)

Table 1. Panel B

Cycle *	Health states			
	No prior MI	Prior MI	Prior MI	Dead
		Preserved LVF	Impaired LVF	
1	0	1000	0	0
2	0	850	50	100
3	0	723	85	193
11	0	197	116	687
21	0	39	46	916
31	0	8	13	979
40 †	0	2	4	994
	0	6657	2195	31 148

Output: number of patients in each health state at the start of selected simulation cycles

LV = left ventricle; LVF = left ventricular function; MI = myocardial infarction

* The cycle-interval length is 1 year

† The patients who were still alive at the end of cycle 40 were supposed to have died just at the start of cycle 41 (i.e. at the virtual age of 100)

At the end of the second year, 723 patients are still alive and have preserved LVF, 85 another have impaired LVF and a total of 193 patients have died. The process continues until a virtual maximum age is reached; in this case for example age 100. After 40 simulation cycles, the total number of life years in the state 'Prior MI, preserved LVF' amounted 6657. Hence, the estimated life expectancy in a condition with preserved left ventricular function is estimated at 6.7 years, while the estimated life expectancy with impaired left ventricular function is estimated at 2.2 years. Total life expectancy is estimated at 6.7 years plus 2.2 years equals 8.9 years. Since simulation started with a population of patients who are copies of Mr. Jones, these results might be used for the management of Mr. Jones as an individual.

The 'classical' Markov model has no 'memory': the past history such as clinical events has no influence on the risk of death or clinical events such as myocardial infarction.[24] Hence, the Markov model assumes that all individuals starting a cycle in a given state are a homogenous group.[25] This fundamental property represents an obvious oversimplification of reality, since the incidence of events as well as treatment choices usually depend on patient characteristics. If one would like to account for these relations, additional health states should be added to the model. However, with each added relation the number of health states roughly doubles, and soon the model becomes quite complex and disorderly, whereas the running time increases rapidly.

Alternatively, memory can be added to the model by making use of tracking variables.[25] When a certain event has occurred in a patient, a virtual marker is attached to this patient, so that in future simulations the risk and time to a consecutive event will be adapted accordingly. Likewise, a large amount of tracking variables adds to excessive model complexity. In conclusion, although it is generally recognized that the Markov model provides a useful tool for modelling processes at population level, its properties limit studying the remaining lifetime of an individual patient. Micro-simulation, also termed discrete-event simulation, Monte Carlo simulation or dynamic risk modelling offers a better solution for this situation.

Micro-simulation

In contrast to Markov models, which simulate the remaining lifetime of a virtual patient population, micro-simulation simulates the remaining lifetime of one single virtual patient at a time and builds a virtual patient population by repeating the simulation numerous times.[26] In the late 1950s, the first practical use of micro-simulation was introduced at the decision-making units for social policy.[6] Gradually, the concept of micro-simulation was used in broader policy questions at different departments.[6] The development of prediction models for health policy questions using micro-simulation has subsequently been increasing.

A virtual CAD patient like Mr. Jones, with his unique risk profile is at risk of several disease related events (myocardial infarction, stroke, etc.) for the remainder of life. For each of the possible events that can happen to a patient, micro-simulation can simulate the age at which these will happen. Patient characteristics influence the occurrence of these events. This is also called stochastic uncertainty.[27] This simulation can be done repeatedly for individual subjects by randomly drawing from the probability distribution of the time to that particular event.[26] The shape and parameters of the distribution are related to the patient's unique profile.

The event with the earliest age of occurrence is then the one that 'really' happens. The patient may or may not survive the event. If he survives, he may or may not receive specific treatment. If he survives treatment, his unique profile is adjusted, since at least his virtual age has increased and his simulated medical history has been altered, and new random times until events are drawn. In practical terms: Mr. Jones started as a healthy 60 years old male who experienced an MI, and was treated with a PCI. The event and treatment altered his unique profile so the probability for having a recurrent event also will change. Further simulations will take these changes into account, and consequently provide the 'memory' in this model. The process continues until the patient dies. If the simulation of the lifetime of this patient is repeated numerous times, a virtual population is created, consisting of patients with identical baseline characteristics and with a broad range of possible outcomes. This dataset can then be used to determine the most likely prognosis of an individual patient with those characteristics. Currently available desktop computers will be able to run this entire process within seconds for simple simulations. Software has been developed to perform micro-simulation in several studies, for instance the global diabetes model[28], the colorectal cancer screening[29] and for aortic valve replacement.[30] A general software package for wider application of micro-simulation still needs development.[31]

The above examples illustrate that both Markov and micro-simulation models are capable of taking serial outcomes into account, contrary to regression. Another similarity between Markov and micro-simulation is that both modelling techniques use input parameters from prior research, such as meta-analyses, or analyses on existing datasets available to the researcher. Micro-simulation has several advantages over a Markov state transition model. Since micro-simulation does not work with predefined cycle lengths, it allows events to occur at any point in time.[25] Furthermore, since patient characteristics need not be defined by certain states in micro-simulation, the model is capable of considering a wide range of individual variations in state of health. Also, micro-simulation allows changing hazards over time.[32] Hazards may change over time because of changes in risk profile, such as increasing age, or because of competing events that occur during follow-up. The main advantage of micro-simulation is that it can be used as a tool for tailored clinical

decision making, since it provides detailed insight into the life history of individuals. The output of the simulation will be sharpened to the individual patient with his unique patient profile at the beginning of the simulation, and take into account all the events occurring over time during simulation. For a particular patient, the expected benefits of treatment can be quantified in terms of numbers of each of the events that will occur during the remaining life, the duration of event-free periods, and the remaining life expectancy.

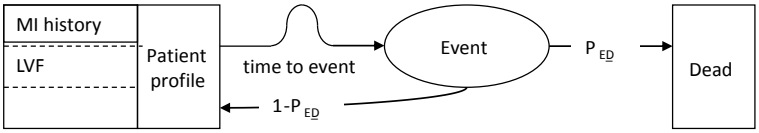

Figure 2. Simple micro-simulation to study the lifetime clinical corse of CAD patients

Figure 2 and Table 2 describe a simple micro-simulation model that is equivalent to the Markov model in Figure 1, and might be used to study the long-term clinical course of Mr. Jones. Specifically, panel A and B in table 2 summarize the input of the micro-simulation model, and panel C summarizes the output. The probabilities chosen in this example are all fictive. Besides the medical history, the patient profile may comprise all kinds of variables which might influence the time to event, without disorganizing the model. We ran this model 1000 times in order to construct a virtual population with identical baseline characteristics, but with varying life courses. In the first run, Mr. Jones died at age 61.3 after having had another MI at age 61.3. His LV function became severely impaired at age 55.0. In the second run, Mr. Jones died at age 83.8 after having had 2 MI's at age 67.0 and 74.4. He had preserved LV function until the end of his life. The average age of death of the 1000 clones of Mr. Jones was 68.8 years. Hence, for this 60 years old male, his estimated life-expectancy was 8.8 years. His life expectancy in a condition with preserved left ventricular function was estimated at 6.4 years.

Table 2, panel A. Occurrence estimates of a simple micro-simulation model to study the lifetime course of 1000 patients of age 60 with prior myocardial infarction and preserved LV function

Event	Patient profile	Probability function	Parameter
MI	All profiles	Exponential	0.05
Impaired LVF	No prior MI	Exponential	0.005
	Prior MI – preserved LVF	Exponential	0.05
	Other profiles	Fixed probability	0
All-cause death	No prior MI	Exponential	0.01
	Prior MI – preserved LVF	Exponential	0.09
	Prior MI – impaired LVF	Exponential	0.135

Input: time to event distributions; the unit of time is year

Table 2, panel B. Mortality risks of the events

Event	Patient profile	Outcome	Probability
MI	No prior MI	Alive, preserved LVF	0.80
		Alive, impaired LVF	0.10
		Dead	0.10
	Prior MI –preserved LVF	Alive, preserved LVF	0.60
		Alive, impaired LVF	0.20
		Dead	0.20
	Prior MI – impaired LVF	Alive, impaired LVF	0.70
		Dead	0.30
Impaired LVF	All profiles	Alive	1
All-cause death	All profiles	Dead	1

Input: immediate outcome after the event

Table 2, **panel C**. Model output

Median (Q1,Q3) time to death, year	6.2 (2.7, 12.2)
Median (Q1,Q3) time to impaired LV function, year	4.8 (1.8, 8.9)
Life expectancy, year	8.8
Life expectancy with preserved LV function, year	6.4

Output: number of patients in each health state at the start of selected simulation cycles

LV = left ventricle, LVF = left ventricular function; MI = myocardial infarction

One of the variables that might influence the time to event is the choice of treatment by physicians. Table 3 shows in more detail how micro-simulation can be helpful to estimate life-long treatment effects in an individual patient. In this particular example, we simulated treatment with a drug that reduces death and myocardial infarction by 20%. Apparently, according to the simulation, the effects on life-expectancy are highly dependent on the patient profile. This kind of data can be used by physicians to decide on treatment, as well as to increase patient awareness of the magnitude of the effects of prescribed medication. Although micro-simulation has several advantages, it also entails practical challenges.[32] First, after the determinants and events of interest have been specified, the parameters of their associations should be assessed. The value and uncertainty of the parameters (beta coefficients) can be estimated using various approaches. Databases at the researcher's disposal may be used. A drawback of this approach is that the results may not be generalizable to patient populations other than the ones used for parameter estimation. Alternatively, literature research and a subsequent meta-analysis or meta-regression could be performed.

Table 3. Micro-simulations of the treatment effects of an hypothetical drug that reduces mortality and myocardial infarction by 20% for different patient profiles

				***Without* treatment**				***With* treatment**				
Age	Prior MI	Preserved LVF	Impaired LVF	Median (Q1,Q3) time to death, years	Life expectancy, years	Median (Q1,Q3) time to impaired LVF, year	Time until impaired LVF, years	Median (Q1,Q3) time to death, years	Life expectancy, years	Median (Q1,Q3) time to impaired LVF, years	Time until impaired LVF, years	Δ life expectancy without -with treatment, years
60	y	y	n	6.7 (3.1, 13.0)	9.1	5.0 (1.9, 9.7)	7.0	7.5 (3.2, 15.0)	10.5	5.0 (2.1, 10.4)	7.6	1.4
60	n	y	n	18.1 (9.4, 30.2)	22.4	16.3 (8.6, 27.6)	20.6	21.4 (11.9, 36.2)	26.7	18.8 (9.9, 32.1)	23.9	4.3
60	y	n	y	4.7 (1.9, 9.1)	6.6	NA	NA	5.7 (2.3, 10.7)	7.8	NA	NA	1.2
70	y	y	n	6.8 (3.0, 12.6)	9.0	5.0 (2.1, 9.5)	7.0	8.2 (3.6, 14.8)	10.7	5.4 (2.2, 10.4)	7.6	1.7
70	n	y	n	17.8 (9.7, 29.9)	21.9	15.1 (8.5, 26.1)	20.0	21.7 (10.9, 37.9)	27.4	17.9 (9.2, 32.5)	24.1	5.5
70	y	n	y	4.6 (1.7, 9.4)	6.8	NA	NA	5.7 (2.4, 11.5)	8.2	NA	NA	1.4
80	y	y	n	6.6 (2.9, 12.6)	9.1	4.7 (1.9, 9.6)	6.7	7.7 (3.3, 15.0)	10.9	5.1 (2.3, 10.2)	7.6	1.8
80	n	y	n	17.8 (9.8, 29.7)	21.9	15.9 (8.3, 26.2)	19.7	21.8 (11.9, 36.9)	26.5	18.3 (9.4, 32.7)	23.6	4.6
80	y	n	y	4.3 (1.8, 9.2)	6.4	NA	NA	5.8 (2.7, 11.4)	8.3	NA	NA	1.9

y = yes; n= no; LVF = left ventricular function; MI = myocardial infarction

This leads to availability of larger groups of subjects, and, since various studies are combined, improved generalizability. Furthermore, a combination of both available databases and literature research may be used. For all of the alternatives the main challenge is how to obtain reliable parameters. Nevertheless, the advantage of micro-simulation is the diversity of patient profiles that can be taken into account, while in RCT's patients characteristics are dependent on inclusion criteria of the trial and may not apply to the specific patient a physician is treating.

Second, the shapes of the associations should be assessed.[33] The most straightforward approach is to assume that the hazards of experiencing the endpoints of interest are constant over time. However, this may be an oversimplification of reality. Alternatively, risk may be assumed to take two phases of constant hazard, with the hazard being greater during the first phase than during the subsequent period. For example, the first months after an MI the risk of having a recurrent event will be higher than later on in time, when risk will stabilize. The concomitant distribution has two periods with two different hazards which can be calculated with the so-called two-period function. Other functions may also be applied to accommodate a changing risk over time. A risk that increases with time may be present with ageing; the risk of having an event will become larger and the hazard ratios should be adjusted. To calculate this, a so-called Weibull function can be applied. This is a generalization of the exponential distribution to accommodate a changing risk over time. In summary, the choice of a particular shape of an association depends on the situation that is being modelled and should be well-considered.

Furthermore, validation forms an essential part of the development of a micro-simulation model as it demonstrates the model's credibility and reliability.[34] In our context, validation refers to the process of determining the extent to which the relationships that have been modelled are able to describe patient prognosis as specified by empirical data. One way to go about this is to utilize a training set and a testing set by randomly dividing the available database into two data sets. The training set is used for developing the model, and the testing set is used to assess model discrimination and calibration. Alternatively, another study population could be used for validation. Finding a study population that has similar characteristics and is consequently suitable for this purpose may be challenging.

Finally, if the objective is to model long-term survival, validating and calibrating the right hand tails of the model may be problematic. We face the intrinsic difficulty that there are often no sufficiently large datasets containing observational data followed-up until death. A solution for this problem is currently not at hand. Thus, we often resort to making assumptions about the risk of clinical events beyond the end of the follow-up of a study.

From the above it is evident that the quality, reliability and usefulness of a simulation model are mainly determined by the quality of the input, which requires empirical data of sufficient quality.

Micro-simulation models have been developed for patients requiring aortic valve replacement, in order to assist with the choice of an appropriate type of valve.[35] Van Geldorp et al. used a predefined group of baseline characteristics which could change after the occurrence of an event.[30] Model parameters were extracted from published literature. Different hazard functions were used depending on the clinical situation; for example, a constant hazard was used to model the probability of an event which is constant over time, while a Weibull function was used to model a risk that increases with time. The micro-simulation model dealt with competing risk by allowing occurrence of events to change the patient's profile, resulting in changes in probability of having a next event. The model that was developed was meant to predict long-term outcome. Since follow-up time after aortic valve replacement was limited in the literature, the model was extrapolated and validated on the investigator's own database that contained a longer follow-up time. Similar models have been developed for prediction of prognosis after aortic valve replacement.[6, 36, 37]

Since the above-described research has been used for the development of new guidelines in the field of aortic valve replacement,[38] it has had direct consequences for clinical practice. The choice of treatment by physicians and the quality of life of patients have been improved. Micro-simulation has had a great share in this improvement, for example by lowering the age threshold for using biological valves.[36] This demonstrates that micro-simulation may also be a promising technique in other medical fields.

CONCLUSION AND PERSPECTIVE

In conclusion, by applying micro-simulation, the most likely prognosis of an individual patient can be calculated by means of a virtual dataset. The remaining lifetime of one single virtual patient is simulated based on the unique individual profile. For each of the possible events that can happen to a patient, micro-simulation can simulate the age at which it will occur. The event with the earliest age of occurrence is then the one that 'really' happens. By repeating this simulation numerous times, and by subsequently building a virtual patient population from the total of these virtual patient lifetimes, the virtual dataset is obtained.

Consequently, the results of a micro-simulation can inform physicians by estimating most likely outcomes regarding a broad range of clinical events in large numbers of virtual patients with equal baseline conditions. Micro-simulation can be used to evaluate treatment effects by estimating the event-free life expectancy without and with treatment. An important advantage of micro-simulation models compared to regression models, is that multiple events, occurring consecutively in time, may be examined. While Markov models may also examine consecutive events, these models have limited possibilities to accommodate changing baseline characteristics. Furthermore, micro-simulation is able to

take into account changing hazards over time, making the model more reliable. The main challenges that should be addressed while constructing a micro-simulation model include the input data requirements, assessment of the shapes and parameters of the associations between the determinants and events of interest, the validation of the model and the often limited follow-up time of the input data.

For readers who wish to read more about micro-simulation, or who wish to bring it into practice, we recommend the following resources. For more information about decision analyses, Markov models and micro-simulation, Hunink et al.[25] is an excellent reference. Rutter et al.[6] give additional insight into the development and application of micro-simulation models for health policy questions. Weinstein et al.[39] have described methodological aspects which can help in evaluating a health-care model. For a practical example of a micro-simulation model that is currently being used, readers may refer to the website of CardioThoracicResearch.[40]

The European Society of Cardiology, the American College of Cardiology and the American Heart Association have established guidelines for the diagnosis and management of cardiovascular diseases. These guidelines aim to support the treating physician by summarising the evidence for the usefulness of treatment that has emerged from clinical research. However, the guidelines for ischemic heart disease, which encompass chronic stable angina and (non)-ST-elevation acute coronary syndromes, contain almost 500 recommendations[41] and it is unrealistic to assume that physicians will be able to adhere to all of these without computer assistance. We anticipate that micro-simulation for decision-making in established CAD may act as an electronic assistant, which helps to ensure that guideline recommendations will be effectively implemented. Furthermore, it may serve as a practical tool that facilitates tailored clinical decision making in individual patients with established CAD in whom optimal management is unclear (for example, when individual patient characteristics differ from those of specific populations on which trial results and recommendations are based).

Such a micro-simulation model would take into account patient characteristics, medical history and medication use (Figure 3).

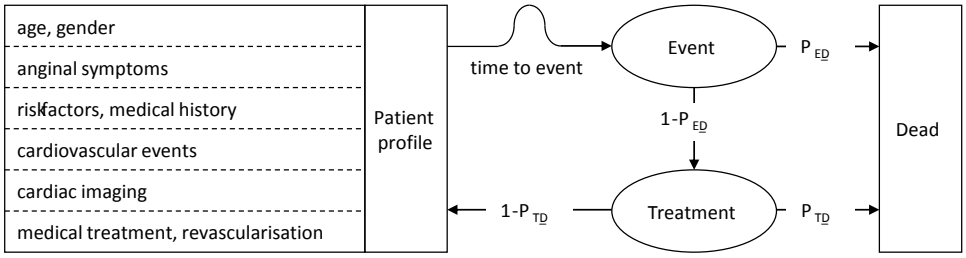

Figure 3. Extended micro-simulation model for tailored decision making in individual CAD patients

Based on these determinants it would provide detailed insight into the life history of an individual patient, and thereby guide treatment decisions for that particular patient. A treatment decision could be added to the patient profile, and subsequently prognosis with or without this treatment could be compared. Thus, a well-grounded treatment decision could be made by the physician.
Such a micro-simulation model could be expandable to the broader range of atherosclerotic diseases. We expect that such a development would ultimately result in improved care and a better life for patients with cardiovascular disease.

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3.2|

generally applicable software package for micro-simulation modeling

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ABSTRACT

Microsimulation can be used to predict the prognosis of an individual patient based on a virtual patient population of copies of that patient.

In this study we compare the outcomes of an existing validated microsimulation program that is designed to study valvular heart disease and a newly developed microsimulation program that is designed to study heart diseases in general.

We studied in depth the results of both systems to model the prognosis of a 40 year old male patient undergoing allograft surgery. Furthermore we studied the model results in relation to age and sex to provide a general overview of the most important outcome variables including operative mortality, average survival time, average event free time and average time to reoperation.

Our results show a good agreement between the two systems regarding all simulations of allograft surgery. We intend to use the newly developed software to explore other disease/event related prognostic models.

INTRODUCTION

One of the simplest forms of microsimulation is the simulation of a coin flip. By using computer software we may throw a coin a large number of times. For an honest coin (i.e. probability of "head" is 0.5; note that parameters can be freely chosen) we should expect to observe approximately the same number of either heads or tails. A more advanced version of this technique can be used in medicine to predict disease prognosis as described below.[1]

Microsimulation in AVR

The use of microsimulation is a widely accepted and useful strategy to support clinicians in choosing an appropriate treatment for patients with an indication for aortic valve replacement (AVR).[2, 3] Microsimulation simulates the probability of operative mortality and AVR related adverse events for an individual patient based on his clinical characteristics and the type of AVR that is being considered. Specifically, it creates a virtual population of patients with identical baseline characteristics, and calculates the event-free period after surgery, reoperations and life expectancy, for each type of AVR.

For each virtual patient, a lifetime of events and reoperations is simulated, using functions and random distributions, based on parameters derived from epidemiological studies.

Consecutive events during the patients' lifecourse are simulated as follows. After the initial operation, the time to each individual valve-related incident is calculated. The event that occurs after the shortest time period is chosen as the one that actually took place in the virtual patient. After this event, a reoperation may be needed or another event may occur. This second event is simulated by repeating the procedure. The simulation for a virtual patient ends when the patient dies, either due to background mortality, event-related mortality or operative mortality.

AVRSim

In the past, our centre has developed software (AVRSim) for simulating the lifecourse of AVR patients to support physicians in choosing the most appropriate type of AVR. This software was validated internally and externally.[4-6]

AVRSim is specifically tailored to handle AVR patients. The software compares the following types of AVR: allograft, bioprothesis, mechanical, and autograft. Unfortunately, the current version of AVRSim could not be easily transformed to other disease/event/treatment related models. AVRSim is available, after registration, for download.[7]

General microsimulation toolkit

We hypothesize that the microsimulation technique can also be applied to other clinical patient populations. For this purpose, we have developed a new software package, General Microsimulation Toolkit (GMT). With this package we aim to provide a microsimulation toolkit that is applicable to any given type of disease (or disease related event) and treatment strategy.

The key features of a disease in the system are the time function to develop the disease (or disease related event), the mortality function for the disease, the adjustment for baseline parameters and the function to determine the most likely treatment. Each treatment has a mortality function (for example the risk of dying during an operative procedure), a time function to determine the most likely time to treatment-related event and the adjustment for baseline parameters.

The GMT is a web based system developed in C# and uses the JEP.NET library[8] for the mathematical functions. The system can handle a variety of statistical methods such as: (logistic) regression models, (log) normal, 2-period, Weibull, Pareto, and Gompertz.

The system has a user-friendly interface to incorporate these statistical methods into a patient-event-treatment microsimulation model. It calculates the time-to-event, event-free period, life expectancy, treatment efficiency and treatment related events. Moreover, it facilitates the comparison of the outcomes of each simulation (statistically and graphically in a survival curve) for different treatment choices.

METHODS

The aim of this study is to compare the results of AVRSim and GMT. In this paper, we consider one case in depth; the case of a 40 year old male undergoing allograft surgery. We also consider different populations with different ages and genders.

Case of a 40 year old male

In this study, we compare the results of a hypothetical case study of a 40 year old male undergoing allograft surgery. We have generated a virtual patient population of 10000 identical individuals in both systems.

In Table 1 we show the parameters of the allograft model. The risk function, together with the age dependency, calculates the time to an AVR related event, of the event occurrence after an allograft procedure.

The mortality risk describes the mortality risk when the event occurs. The reoperation risk is the risk of undergoing a reoperation after the event. The reoperation type describes the reoperation which is performed after the event.

Table 1. Parameters of the allograft microsimulation model used in both systems

Event	Risk function	Age dep.	Mortality Risk	Reoperation risk	Reoperation
Valve thrombosis	Zero-risk	0	0	1	Allograft
Thromboembolism	Exponential(0,006)	0	0.1	0	
Hemorrhage	Exponential(0,001)	0	0.07	0	
Non-structural dysfunction	Exponential(0,005)	0	0	1	Mechanical
Endocarditis	Exponential(0,005)	0	0.25	1	Allograft
Structural dysfunction	Weibull(2,234,;3,669)	0,0112	0	1	Mechanical

The operative mortality odds for an allograft at age 40 is 0.0260; the odds ratio (OR) for age (per year) is 0.0218; the OR per reoperation is 0.5306. We used a background mortality based on the Dutch life tables and a hazard ratio of 3.65.

Different patient populations

In a second analysis we constructed different virtual patient populations consisting of men and women with ages defined by 10-year age-intervals (n=10000, age-range 10 to 70). We simulated an allograft replacement operation in each population. We analyzed operative mortality, the average survival, the average freedom of event time and the average freedom of reoperation time.

We used similar parameters for an allograft as described in table 1 and subheading 2.1. However the operative mortality is different because it is age dependent. The hazard ratio used to calculate the background mortality is age and gender dependent. Again, we use the Dutch life tables for the background mortality.

RESULTS

Results case of a 40 year old male

Negligible differences were present in the average survival in years after the allograft procedure, average event free period, average number of persons free of reoperation, number of event free persons, number of individuals that didn't receive a reoperation, the mortality of the initial allograft procedure and the number of individuals that died due to non-valve related events/operations ('mortality non related').

Table 2 also shows also the number of events for each event. In AVRsim, 1600 individuals developed a thromboembolism, 177 individuals developed 2 thromboembolisms and 13 individuals developed 3 thromboembolisms.

Table 2. Results of a case study of 40 year old male after allograft surgery in AVRSim and GMT.

	AVRSim	**GMT**
Survival (years)	21.17	21.30
Event free (years)	10.70	10.76
Reoperation free (years)	11.11	11.41
Number of event free	1670	1683
Number of reoperation free	1937	2073
Mortality first operation	242	234
Mortality non-related	8218	8235
Valve thrombosis		
1	46	44
Thromboembolism		
1	1600	1556
2	177	144
3	13	13
4	0	1
Hemorrhage		
1	1337	1378
2	203	144
3	23	12
4	3	1
Non structural dysfunction		
1	878	878
2	63	54
3	1	2
Endocarditis		
1	575	631
2	29	23
3	2	0
Structural dysfunction		
1	7326	7144

Results different patient populations

Table 3 shows the results for the populations of patients undergoing allograft operation that we generated. For each population, we simulated age from 10 to 70 for both genders. The table shows the most relevant outcome parameters; percentage of operative mortality of the allograft operation ('Op. mortality'), average survival time in years, average time to first event ('Event free') and average time to first reoperation ('Reoperation free'). The last row displays the range of the maximum difference between each of the columns.

Due to the fact that AVRSim uses a non-seeded random function, the results are always the same. This is clearly shown by the identical operative mortality for both genders.

Table 3. The results of AVRSim vs. GMT for different virtual populations with different initial age in years and gender (M for male and F for female). Op. mortality is the percentage of the population that died during the initial allograft procedure.

	Gender	Op. mortality (%)		Survival (years)		Event free (years)		Reoperation free (years)	
		AVRSim	GMT	AVRSim	GMT	AVRSim	GMT	AVRSim	GMT
10	M	1.38	1.15	41.34	41.60	8.49	8.48	8.74	8.94
	F	1.38	1.45	44.35	44.30	8.51	8.47	8.76	8.96
20	M	1.55	1.50	33.58	33.60	9.27	9.25	9.57	9.81
	F	1.55	1.42	36.51	36.55	9.35	9.37	9.66	9.97
30	M	1.94	2.09	26.77	26.98	10.12	10.01	10.48	10.72
	F	1.94	2.06	28.62	28.62	10.14	10.09	10.50	10.76
40	M	2.42	2.34	21.17	21.30	10.70	10.76	11,11	11.41
	F	2.42	2.30	22.67	22.84	10.73	10.75	11,14	11.42
50	M	2.97	3.07	16.77	16.82	10.76	10.77	11.20	11.37
	F	2.97	3.12	18.78	18.90	11.15	11.04	11.61	11.76
60	M	3.58	3.88	13.08	12.92	10.05	9.93	10.45	10.43
	F	3.58	3.77	14.41	14.41	10.76	10.72	11.2	11.36
70	M	4.46	4.72	9.87	9.77	8.52	8.41	8.82	8.75
	F	4.46	4.60	10.29	10.37	8.93	8.91	9.25	9.32
Range diff		-0.3 ; 0.23		-0.26 ; 0.16		-0.06 ; 0.12		-0.31 ; 0.07	

DISCUSSION AND CONCLUSIONS

We have demonstrated good agreement between AVRSim and GMT. We conclude that the underlying mathematical functions are correctly implemented. The AVRSim models have been internally and externally validated. With the current study, the GMT model for AVR has been validated against the AVRSim AVR model.

The next step is the application of this software to other prognostic models, including cardiovascular diseases and treatments. Obviously, these new models need to be evaluated internally and externally before they can be used in daily practice.

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PART III |

The role of inflammation in prediction of clinical outcome in coronary artery disease

4

Inflammatory biomarkers, coronary atherosclerosis and clinical outcome

14

Inflammatory biomarkers,
coronary atherosclerosis
and clinical outcome

4.1 |

Cytokines, VH-IVUS derived extent and composition of coronary atherosclerosis, and major adverse cardiac events

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ABSTRACT

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

Methods: Between 2008-2011, IVUS(-VH) imaging of a non-culprit coronary artery was performed in 581 patients (stable angina pectoris (SAP), n=261; acute coronary syndrome (ACS), n=309) undergoing coronary angiography from the ATHEROREMO-IVUS study. Plaque burden, presence of VH-IVUS-derived thin-cap fibroatheroma (TCFA) lesions, and presence of VH-TCFA lesions with plaque burden ≥70% were assessed. Blood samples for cytokine measurement were drawn from the arterial sheath prior to the angiography procedure. We applied linear and logistic regression.

Results: TNF-α levels were positively associated with plaque burden (beta (β) [95%CI]: 4.45 [0.99-7.91], for highest vs lowest TNF-α tertile) and presence of VH-TCFA lesions (odds ratio (OR) [95%CI] 2.30 (1.17-4.52), highest vs lowest TNF-α tertile) in SAP patients. Overall, an inverse association was found between IL-10 concentration and plaque burden (β [95%CI]: -1.52 [-2.49- -0.55], per Ln(pg/mL) IL-10) as well as IL-10 and VH-TCFA lesions with plaque burden ≥70% (OR: 0.31 [0.12-0.80],highest vs lowest IL-10 tertile). These effects did not reach statistical significance in the separate SAP and ACS groups.

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

INTRODUCTION

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

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

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

METHODS

Study population

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

ethics committee of Erasmus MC, Rotterdam, the Netherlands. Written informed consent was obtained from all included patients and the study protocol conforms to the ethical guidelines of the Declaration of Helsinki.

Biomarkers

Blood samples were drawn from the arterial sheath prior to the diagnostic coronary angiography or PCI procedure, and were available in 570 patients for the current study. The blood samples were transported to the clinical laboratory of Erasmus MC for further processing and storage at a temperature of -80 ^{0}C within two hours after blood collection. C-reactive protein (CRP) was measured in serum samples using a immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Cobas 8000 modular analyzer platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland). These analyses were performed in the clinical laboratory of Erasmus MC.

Frozen EDTA-plasma samples were transported under controlled conditions (at a temperature of -80^{o}C) to Myriad RBM, Austin, Texas, USA, where the concentrations of TNF-α, TNF-β, TNF R2, INFγ, IL-6, IL-8, IL-10 and IL-18 were determined using a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, Texas, USA). While TNF-α, TNF R2, IL-6, and IL-8 were determined in the full cohort of 570 patients, TNF-β, INFγ, IL-10 and IL-18, were determined in a random subset of 473 patients. This difference in numbers resulted from batch-wise handling of the samples in combination with an update of the composition of the multiplex assay by the manufacturer in-between two batches. None of the biomarker laboratories had knowledge of clinical or intracoronary imaging data.

Intravascular ultrasound

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

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

Statistical analysis

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

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

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

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

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

RESULTS

Baseline characteristics

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

Biomarkers and extent of atherosclerosis

The results of the analyses for plaque burden of the entire measured segment are shown in Figure 1 and supplemental tables 1a,b and c. Higher TNF-α was associated with higher coronary plaque burden in patients with SAP (β [95%CI]: 4.45 [0.99-7.91], for the highest vs the lowest tertile of TNF-α). Such an effect could not be demonstrated in patients with ACS. Furthermore, lower IL-10 concentrations were associated with higher coronary plaque burden in the full cohort (β [95%CI]: -3.88 [-6.00- -1.76], for the highest vs the lowest tertile of IL-10). This effect was driven by both the SAP patients and the ACS patients. Although effect estimates for the highest tertile of IL-10 were similar in both groups (SAP: -2.95 [-6.23-0.33], ACS: -3.42 [-6.57- -0.27], in the SAP patients the estimates, as well as the linear trend, did not reach statistical significance.
After multivariable adjustment, associations remained essentially the same for both TNF-α and IL-10.

Table 1. Baseline characteristics.

	Total (n=570)	ACS patients (n=309)	SAP patients (n=261)
Patient characteristics			
Age, years (mean±SD)	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Men, 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, 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 renal insufficiency, n (%)	32 (5.6)	13 (4.2)	19 (7.3)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
Procedural characteristics			
Indication for coronary angiography			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	0 (0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0)
Unstable angina pectoris, n(%)	150 (26.3)	150 (48.5)	0 (0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0)	261 (100)
Coronary artery disease			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.4)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
IVUS characteristics			
Segment length (mm), median (IQR)	44.1 (33.7-55.4)	43.9 (32.9-54.1)	44.8 (34.2-57.2)
Plaque burden (%), median (IQR)	39.2 (30.0-46.4)	37.2 (28.0-45.5)	40.2 (31.8-47.8)
Presence of VH-TCFA, n(%)	239 (41.9)	140 (45.3)	99 (37.9)
Presence of VH-TCFA with PB ≥ 70%, n(%)	69 (12.1)	32 (10.4)	37 (14.2)
Serum biomarker concentrations			
C-reactive protein (mg/L), median (IQR)	2.1 [0.8-5.3]	2.8 [1.1-7.0]	1.5 [0.6-3.1]
Tumor Necrosis Factor α (pg/mL) median (IQR)$^{+}$	2.0 [1.4-2.9]	1.8 [1.4-2.6]	2.0 [1.4-3.3]
Tumor Necrosis Factor β (pg/mL) median (IQR)†§	35.0 [18.0-116.0]	20.5 [16.5-44.3]	36.5 [27.0-152.8]
Tumor necrosis factor receptor 2 (ng/mL) median (IQR)$^{\#}$	4.5 [3.6-5.7]	4.4 [3.5-5.8]	4.5 [3.7-5.6]
Interferon γ (pg/mL) median (IQR)*§	5.1 [3.9-7.3]	4.8 [3.8-6.6]	5.7 [4.2-8.2]
Interleukin-6 (pg/mL) median (IQR)$^{-}$	3.5 [2.2-5.8]	3.7 [2.5-6.8]	2.5 [2.1-4.1]
Interleukin-8 (pg/mL) median (IQR)$^{\#\S}$	8.9 [6.8-12.0]	9.9 [7.1-12.6]	8.3 [6.5-10.3]
Interleukin-10 (pg/mL) median (IQR)$^{\#\S}$	5.2 [3.6-9.4]	6.9 [4.1-15.0]	4.4 [3.0-6.0]
Interleukin-18 (pg/mL) median (IQR)*	171.0 [132.3-215.0]	173.0 [133.0-216.3]	169.5 [130.5-211.3]

*Measurable in all patients
$^{\#}$Measurable in >99% of patients, too low to detect in <1%
$^{+}$Measurable in 76% of patients, too low to detect in 24%
$^{-}$ Measurable in 38% of patients, too low to detect in 62%
†Measurable in 8% of patients, too low to detect in 92%
§ TNFβ, IFNγ, IL-10 and IL-18: total n= 473, ACS n=309, SAP n= 261

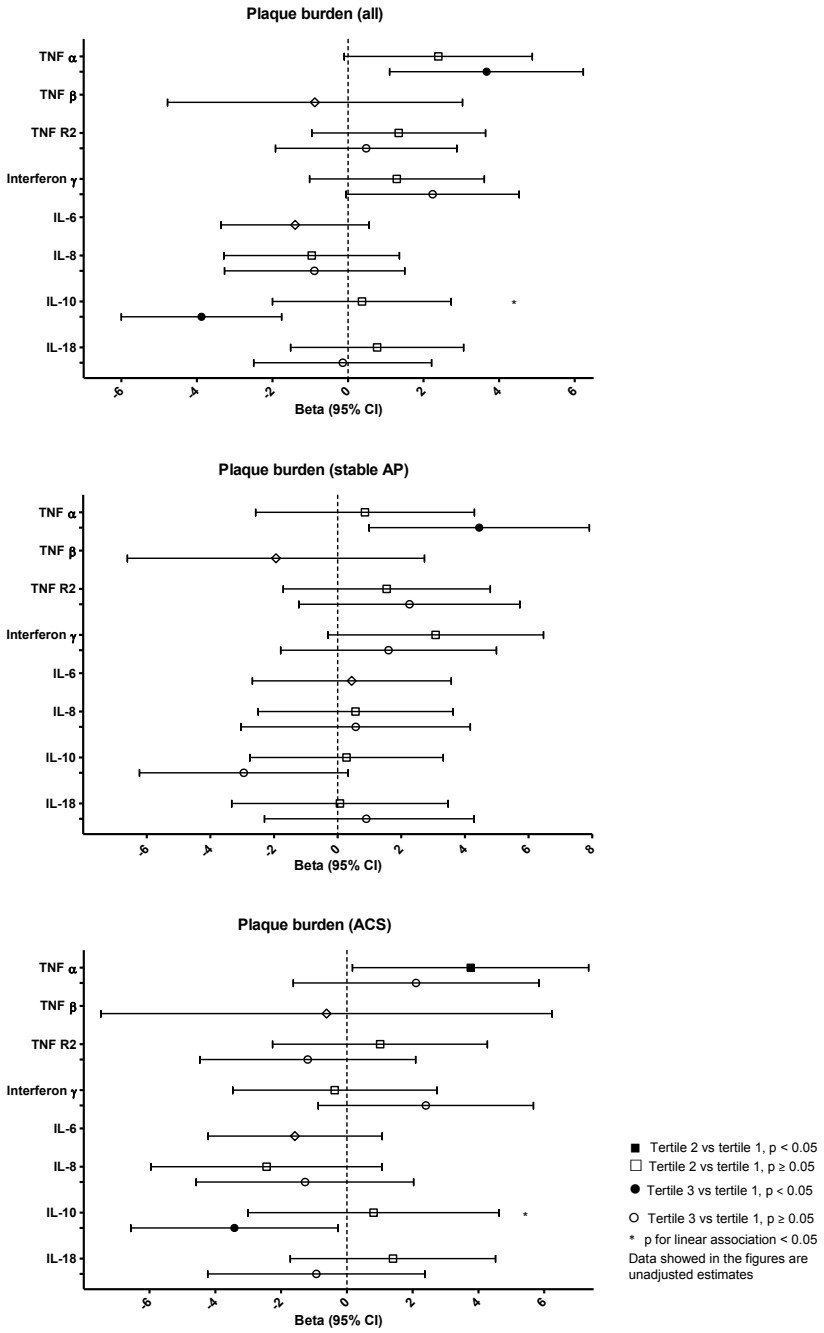

Figure 1. Association of TNF-α, TNF-β, TNF R2, INFγ, IL-6, IL-8, IL-10 and IL-18 with segment plaque burden in all patients, patients with stable AP and patients with ACS.

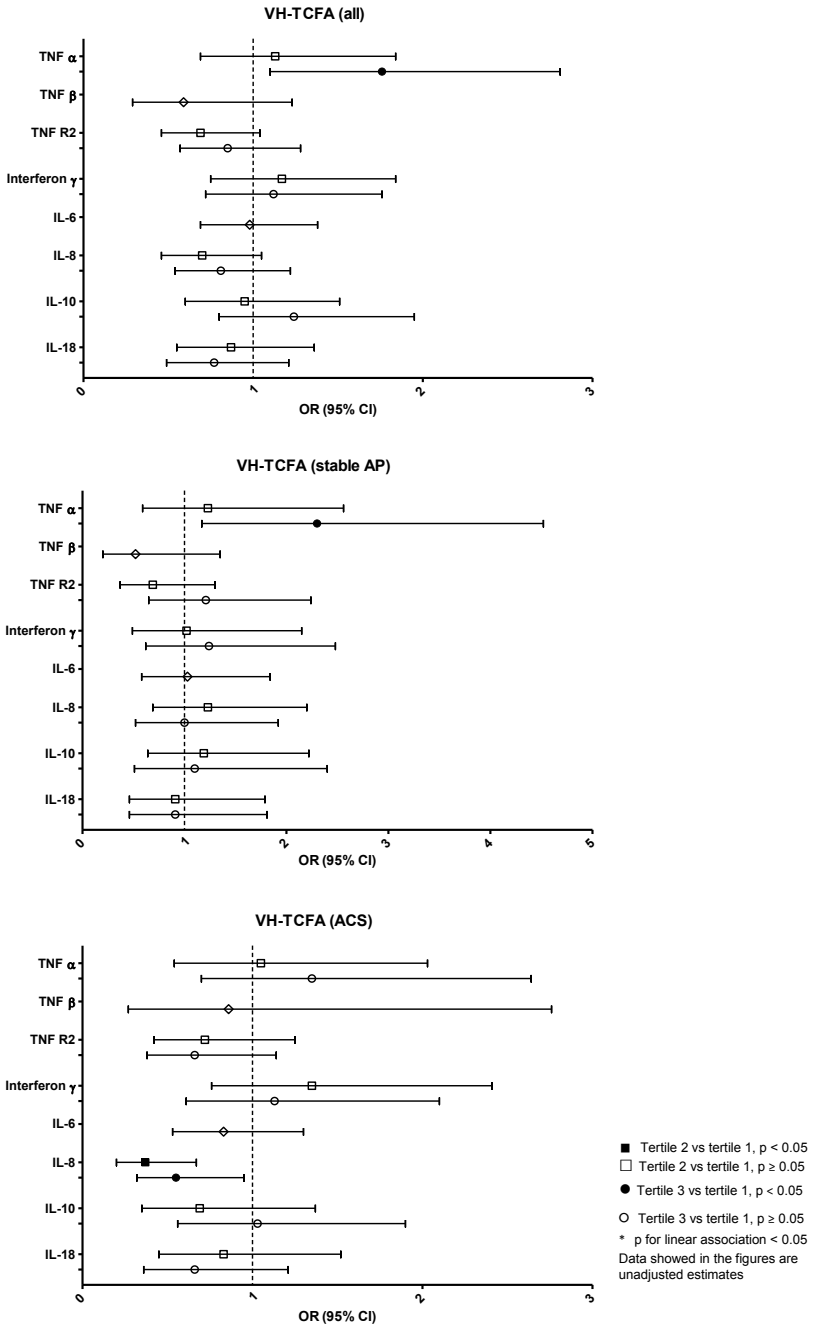

Figure 2. Association of TNF-α, TNF-β, TNF R2, INFγ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA in all patients, patients with stable AP and patients with ACS.

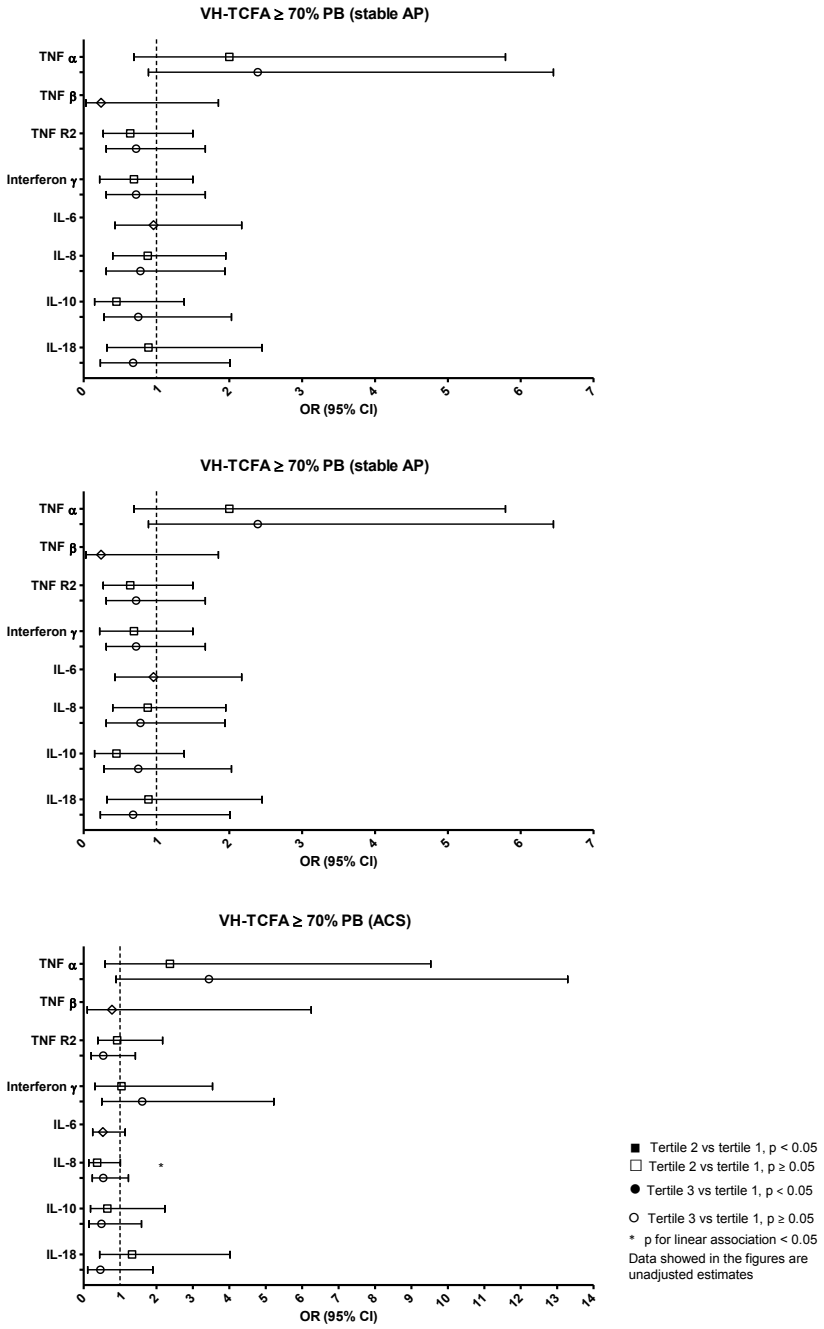

Figure 3. Association of TNF-α, TNF-β, TNF R2, INFγ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA with plaque burden ≥ 70% in all patients, patients with stable AP and patients with ACS.

Biomarkers and composition of atherosclerosis

The results of the analyses for VH-TCFA lesions are displayed in Figure 2 and supplemental tables 2a, b and c. High TNF-α was positively associated with presence of VH-TCFA lesions in patients with SAP (OR[95%CI]: 2.30 [1.17-4.52] for the highest vs the lowest tertile of TNF-α). Such an effect was absent in patients with ACS. Furthermore, higher IL-8 seemed to confer lower risk of VH-TCFA in ACS patients; however, this effect was mainly driven by tertile 2. No associations were present between any of the other biomarkers and VH-TCFA. Higher TNF-α was positively associated with presence of VH-TCFA lesions with a plaque burden ≥ 70% in the full cohort (OR[95%CI]: 2.85 [1.28-6.31] for the highest vs the lowest tertile of TNF-α) (table 4). This effect was driven by both patients with SAP and patients with ACS. Although the effect estimate reached statistical significance in the full cohort, this was not the case in the SAP and ACS groups. Nevertheless, the effect estimates for the highest tertile of TNF-α were similar in magnitude in both groups (SAP: 3.44 [0.89-13.29], ACS: 2.39 [0.89-6.45]. Higher IL-10 displayed an inverse association with presence of VH-TCFA lesions with a plaque burden ≥ 70% in the full cohort (OR[95%CI]: 0.31 [0.12-0.80] for the highest vs the lowest tertile of IL-10, p for trend=0.037). Again, effect estimates did not reach statistical significance in these separate groups. After multivariable adjustment, associations remained essentially the same.

DISCUSSION

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

Inflammation is known to play a major role in atherosclerosis. In a previous study in the current patient population, we have demonstrated an association between CRP and IVUS characteristics (12). TNF-α is a proinflammatory cytokine that is secreted from activated innate immunity cells and is capable of inducing a cascade with a broad range of effects, including immunological activation, apoptosis, and procoagulative and antifibrinolytic actions, all of which can have an effect on the course of atherosclerosis (5, 13). Experimental studies on the role of TNF-α in plaque development and stability in mice have rendered inconsistent results, some finding anti-atherogenic effects and others finding pro-

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

IL-10 is an anti-inflammatory cytokine that is produced by macrophages and lymphocytes (6). This cytokine is capable of inhibiting many cellular processes that may play an important role in atherosclerotic lesion development and in the modulation of plaque composition (6, 21). Mallat et al. (21) investigated atherosclerotic lesions in IL-10 deficient mice and showed increased infiltration of inflammatory cells, increased production of INF-γ, and decreased collagen content, which resulted in development of atheromatous lesions with signs of increased vulnerability. Several clinical studies have been performed on IL-10 and cardiovascular disease. Heeschen et al. (22) demonstrated that a reduced serum IL-10 level in patients with ACS is indicative of a poor prognosis. Most subsequent studies on the association of elevated circulating IL-10 levels with cardiovascular outcome have demonstrated positive associations with better prognosis (23-27). In line with this, we found an inverse association between IL-10 and coronary plaque burden as well as between IL-10 and presence of large, vulnerable plaques (i.e., VH-TCFA lesions with a plaque burden ≥ 70%) in the overall study population. However, we did not find an association of IL-10 with presence of TCFA lesions in general. These results suggest that IL-10 may in particular be associated with lower extent of coronary atherosclerosis and slower growth of VH-TCFAs. In any case, these findings further support the hypothesis of a protective role of IL-10 in atherosclerosis.

The associations of TNF-α and IL-10 with extent and composition of atherosclerosis demonstrated in the current study, suggest that these cytokines may potentially be useful for clinical risk stratification with regard to cardiovascular outcome. However, we did not

find any associations between the cytokines we investigated and major adverse cardiac events during 1-year follow-up, adjusting for clinical covariates (supplemental table 4a-c). Possible explanations may include the fact that the magnitude of the effects of TNF-α and IL-10 may be relatively small in the context of this multifactorial disease, or that the current study lacks statistical power to expose these effects.

We did not find any associations between several cytokines we examined and the extent or composition of atherosclerosis. Analysis of some of the biomarkers (TNF-β and IL-6) was complicated by the fact that over 50% of the measurements were too low to detect. Cytokine assays are generally known to display limitations in terms of % detectability (28, 29). This makes clinical investigations into the pathophysiological role and the prognostic value of these biomarkers challenging. In line with this, few clinical studies have been performed on circulating TNF-β. Furthermore, IL-6 is known to have large circadian variations, and a relatively short half-life of less than 6 hours (30) which also makes this marker difficult to investigate. Clinical studies on circulating TNFR2, INFγ, and IL-8 in patients with coronary artery disease are also limited in number. IL-18 has been examined more often, and has been suggested to be associated with the presence and severity of coronary atherosclerosis (31, 32). In the present study, we could not demonstrate such an association.

Some aspects of this study warrant consideration. Our study population consisted of patients with SAP as well as patients with ACS. The group of patients with ACS is likely to be more heterogeneous, which may have influenced the findings. To account for this, we have performed the analyses separately in both groups. Furthermore, VH-IVUS imaging took place of a prespecified single target segment of a single non-culprit coronary artery, based on the assumption that such a non-stenotic segment adequately reflects coronary wall pathophysiology of the larger coronary tree. Although this assumption may be debated, previous studies evaluating IVUS have demonstrated that the coronary wall of comparable non-culprit, non-stenotic segments of a single vessel does reflect coronary disease burden at large and is associated with subsequent cardiovascular outcome (33-35). Moreover, it is important to note that IVUS is formally not capable of detecting the most rupture prone of all plaque phenotypes, the TCFA (36, 37), because the spatial resolution of IVUS is insufficient for thin cap detection (23, 24). Nonetheless, a concept of VH-IVUS derived TCFA has been postulated for plaques with a plaque burden ≥ 40% and a confluent necrotic core ≥ 10% in direct contact with the lumen in at least three VH-IVUS frames (13, 23). Notably, we have recently demonstrated that such VH-IVUS derived TCFA lesions are strongly and independently predictive of the occurrence of major adverse cardiac events within the current study population (33).

In conclusion, in patients undergoing coronary angiography, higher circulating TNF-α was associated with higher plaque burden and with presence of VH-TCFA lesions in patients

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

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4.2|

Chemokines, VH-IVUS derived extent and composition of coronary atherosclerosis, and major adverse cardiac events

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In press Biomarkers

ABSTRACT

Introduction: This study aims to investigate the relations of several circulating chemokines with the extent and phenotype of coronary atherosclerosis as determined in-vivo by intravascular ultrasound (IVUS) and with 1-year clinical outcome.

Background: Chemokines are involved vascular inflammation and progression of atherosclerosis.

Methods: Between November 2008 and January 2011, IVUS imaging of a non-culprit coronary artery was performed in 570 patients who underwent coronary angiography for acute coronary syndrome (ACS) (n=309) or stable angina pectoris (SAP) (n=261). Subsequent events during 1-year follow-up were adjudicated to be either culprit lesion-related (n=11), non-culprit lesion related (n=27) or indeterminate (n=18).

Results: Higher plasma monocyte chemoattractant protein-1 (MCP-1) (p=0.002 in SAP patients), macrophage inflammatory protein-1α (MIP-1α) (p=0.001) and lower Regulated upon Activation Normal T cell Expressed and Secreted (RANTES) (p=0.025 in ACS patients) were associated with higher coronary plaque burden. Higher MCP-1 (p=0.045 in SAP patients) and MIP-1α (p=0.021) were associated with higher necrotic core fraction. Higher MCP-1 (p=0.052 in SAP patients), higher MIP-1α (p=0.021) and lower RANTES (p=0.067) were associated with the presence of thin-cap fibroatheroma (TCFA) lesions. RANTES was independently associated with the composite endpoints of non-culprit related or indeterminate all-cause mortality, ACS or coronary revascularization (HR per SD increase in ln-RANTES 0.71, 95%CI 0.53-0.96) and non-culprit related or indeterminate all-cause mortality or ACS only (HR per SD increase in ln-RANTES 0.64, 95%CI 0.44-0.94).

Conclusions: Higher plasma MCP-1, MIP-1α, and lower RANTES concentrations are associated with a higher extent, a more advanced phenotype and a higher vulnerability of coronary atherosclerosis. RANTES is a promising biomarker that is independently, inversely associated with occurrence of cardiac events, particularly of death and ACS.

INTRODUCTION

Inflammation has been recognized as an important contributing factor in all phases of atherosclerosis.(1-3) In particular, inflammation is believed to play a crucial role in the development and rupture of vulnerable plaques, resulting in major cardiovascular problems such as myocardial infarction and stroke.(1-3) Circulating inflammatory biomarkers may potentially improve prognostication of patients with atherosclerotic cardiovascular disease.(4)

Chemokines are involved in the recruitment of various leukocytes, such as monocytes, macrophages and T lymphocytes, into the atherosclerotic plaque.(5, 6) Monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β and regulated upon activation normal T cell expressed and secreted (RANTES) are typical C-C motif chemokines that have been studied extensively.(5, 6) Several studies have shown that these chemokines have an important role throughout the entire atherosclerotic process from atherogenesis to plaque destabilization.(5, 6) However, their clinical utility as biomarker remains unclear.(5, 6) Furthermore, prospective data on associations of these biomarkers with in-vivo measurements of extensiveness, phenotype and vulnerability of coronary atherosclerosis is currently lacking. This study aims to evaluate the usefulness of MCP-1, MIP-1α, MIP-1β and RANTES by investigating their relations with intravascular ultrasound (IVUS)-derived measures of coronary plaque burden, quantity of necrotic core, and presence of thin-cap fibroatheroma lesions, and by investigating their prognostic value for major adverse cardiac events.

METHODS

Study population

The design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study has been described elsewhere.(7) In brief, 1098 patients who underwent diagnostic coronary angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS) or stable angina pectoris have been enrolled in ATHEROREMO-IVUS. The ATHEROREMO-IVUS study population consists of 330 patients who were previously enrolled in the randomised double-blinded Integrated Biomarker and Imaging Study-2 (IBIS-2) trial from November 2005 to August 2006 and of 768 patients who were additionally included at Erasmus MC, Rotterdam, the Netherlands from November 2008 to January 2011. Blood samples were drawn prior to the coronary catheterization procedure and IVUS imaging of a non-culprit coronary artery was performed subsequent to angiography. Baseline characteristics of the included patients were prospectively entered into a dedicated database. This study has been approved by the human research ethics committee of

Erasmus MC, Rotterdam, the Netherlands. Written informed consent was obtained from all participants.

Patients were included in the current study analyses when the following criteria were met: 1. not participating in the IBIS-2 trial; 2. IVUS of a non-culprit coronary artery was performed; and 3. plasma samples were available for biomarker measurements. Patients participating in the IBIS-2 trial were excluded from the present analysis in order to prevent possible treatment interaction from the study drug darapladib, which was found to prevent necrotic core expansion as measured by IVUS.(8) In patients who were additionally included at Erasmus MC (n=768), IVUS of a non-culprit coronary artery was performed in 581 patients. In these patients, blood samples were available in 570 patients.

Biomarkers

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

Intravascular ultrasound

Following the standard coronary angiography or PCI procedure, IVUS data were acquired in a non-culprit coronary vessel. Selection of the non-culprit vessel was predefined in the study protocol. The order of preference for selection of the non-culprit vessel was: 1. left anterior descending (LAD) artery; 2. right coronary artery (RCA); 3. left circumflex (LCX) artery. All IVUS data were acquired with the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, CA, USA) using a Volcano Eagle Eye Gold IVUS catheter (20 MHz). An automatic pullback system was used with a standard pull back speed of 0.5 mm per second. The IVUS images were analyzed offline by an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands) that had no knowledge of clinical data. The IVUS gray-scale and IVUS radiofrequency analyses, also known as IVUS virtual histology, were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, CA, USA) software. The external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40 mm). Extent and phenotype of the atherosclerotic plaque were assessed. Plaque burden was defined as plaque and media cross-sectional area divided by external elastic membrane cross-sectional area and is presented as a percentage. The composition of the atherosclerotic plaque was characterized into 4 different tissue types: fibrous, fibro-fatty, dense calcium and necrotic core.(9) A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least 3 consecutive frames. A thin-

cap fibroatheroma (TCFA) lesion was defined as a lesion with presence of >10% confluent necrotic core in direct contact with the lumen.(10, 11) TCFA lesions with a plaque burden of at least 70% were classified as large TCFA lesions.

Study endpoints

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

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

STATISTICAL ANALYSIS

The distributions of the continuous variables, including biomarker levels and the IVUS parameters, were tested for normality by visual examination of the histogram. Normally distributed continuous variables are presented as mean ± standard deviation (SD), while non-normally distributed continuous variables are presented as median and interquartile range (IQR). MCP-1, MIP-1α, MIP-1β and RANTES concentrations were not normally

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

Patients lost to follow-up were considered at risk until the date of last contact, at which time-point they were censored. Cumulative event rates were estimated according to the Kaplan-Meier method. Cumulative Kaplan-Meier event curves were compared by log-rank test. Cox proportional hazards regression analyses were performed to evaluate the relationship between biomarker concentration and clinical endpoints. Biomarkers that were significantly associated with occurrence of MACE in univariable analysis were further evaluated in multivariable analyses. The variables age, gender, diabetes mellitus, hypertension, history of MI and indication for coronary angiography were considered as potential confounders and were entered into the full model. These covariates were a priori chosen, taking into account the number of events available. The final results are presented as hazard ratio (HR) per SD increase in ln-transformed biomarker concentration with 95% CI. All statistical analyses were primarily performed in the overall study population. Heterogeneity in effect estimates between patients with ACS and patients with stable angina were examined using the Z-test for heterogeneity. If there was no heterogeneity, conclusions were based on the effect estimates belonging to the total study population. If there was significant heterogeneity between patients admitted with and without ACS, conclusions were based on effect estimates of the separate groups.

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

Table 1. Baseline characteristics

	Total (n=570)	ACS patients (n=309)	SAP patients (n=261)
Patient characteristics			
Age, years	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Men, 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, n (%)	293 (51.4)	140 (45.3)	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 renal insufficiency, n (%)	32 (5.6)	13 (4.2)	19 (7.3)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
Procedural characteristics			
Indication for catheterization			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	0 (0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0)
Unstable angina pectoris, n (%)	150 (26.3)	150 (48.5	0 (0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0)	261 (100)
Coronary artery disease			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.4)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0%)
Serum biomarker concentrations			
MCP-1, pg/ml *	91 [70-122]	92 [70-133]	88 [71-111]
MIP-1α, pg/ml †	16.0 [12.0-21.9]	15.0 [12.0-21.9]	17.0 [12.0-21.9]
MIP-1β, pg/ml *	123 [92-165]	130 [95-179]	114 [89-146]
RANTES, ng/ml ‡	11.0 [6.4-19.0]	14.0 [7.6-23.0]	9.1 [5.0-14.3]
IVUS segment characteristics			
Imaged coronary artery			
Left anterior descending, n (%)	204 (35.8)	117 (37.9)	87 (33.3)
Left circumflex, n (%)	190 (33.3)	107 (34.6)	83 (31.8)
Right coronary artery, n (%)	176 (30.9)	85 (27.5)	91 (34.9)
Segment length, mm	44.1 [33.7-55.4]	43.9 [32.9- 54.1]	44.8 [34.2-57.2]
At least 1 TCFA	239 (41.9)	140 (45.3)	99 (37.9)
At least 1 TCFA with PB≥70%	69 (12.1)	32 (10.4)	37 (14.2)

* Measurable in >99% of patients; below limit of detection in <1% of patients.
† Measurable in 84% of patients; below limit of detection in 16% of patients.
‡ Measurable in all patients.

ACS indicates acute coronary syndrome; CABG, coronary artery bypass grafting; MCP-1, monocyte chemoattractant protein-1; MI, myocardial infarction; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β; PB, plaque burden; PCI, percutaneous coronary intervention; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris; TCFA, thin-cap fibroatheroma.

RESULTS

Baseline characteristics

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

Associations with coronary atherosclerosis

In patients who were admitted with stable angina pectoris, higher plasma MCP-1 concentrations were associated with higher coronary plaque burden (per SD increase of ln-transformed MCP-1: β=2.56, 95% CI 0.91-4.21, p=0.002) and a higher fraction of plaque consisting of necrotic core (per SD increase of ln-transformed MCP-1: β=1.14, 95% CI 0.02-2.25, p=0.045) (Table 2). Higher MCP-1 concentrations also seemed to be associated with the presence of TCFA lesions (OR per SD increase in ln-transformed MCP-1 1.90, 95% CI 1.00-3.61, p=0.052) in patients who were admitted with stable angina pectoris (Table 3).
Higher MIP-1α concentrations were associated with higher plaque burden (per SD increase of ln-transformed MIP-1α: β=1.66, 95% CI 0.72-2.61, p=0.001), higher necrotic core fraction (per SD increase of ln-transformed MIP-1α: β=0.89, 95% CI 0.23-1.55, p=0.008) and with the presence of TCFA lesions with plaque burden ≥70% (OR per SD increase in ln-transformed MIP-1α 1.75, 95% CI 1.09-2.81, p=0.021) in the total study population.
In patients who were admitted with ACS, lower RANTES concentrations were associated with higher plaque burden (per SD increase of ln-transformed RANTES: β=-1.57, 95% CI -2.94;-0.20, p=0.025) (Figure 1). Furthermore, lower RANTES concentrations also seemed to be associated with the presence of TCFA lesions with plaque burden ≥70% in the overall patient population (OR per SD increase in ln-transformed RANTES 0.76, 95% CI 0.57-1.02, p=0.067).

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

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

P-values were obtained with linear regression analyses with continuous ln-transformed biomarker concentration as independent variable.

ACS indicates acute coronary syndrome; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris.

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

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

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

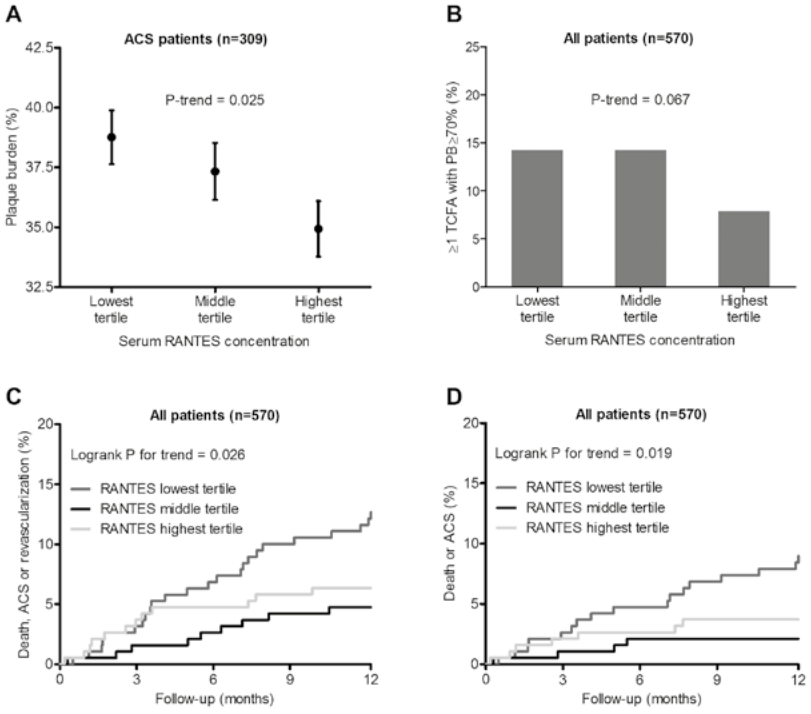

Figure 1.

Major adverse cardiac events

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

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

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

Hazard ratios are per standard deviation increase in ln-transformed biomarker concentration.
MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; MIP-1β, macrophage inflammatory protein-1β;
RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP, stable angina pectoris.

Associations with non-culprit lesion related and indeterminate events

In univariable analysis, RANTES (HR per SD increase of ln-transformed RANTES 0.67, 95% CI 0.50-0.89, p=0.005) was associated with occurrence of the primary endpoint of non-culprit lesion related and indeterminate MACE during follow-up (Table 4). There was no heterogeneity in the hazard ratio estimate between ACS patients and patients with stable angina (heterogeneity p=0.39). RANTES (HR per SD increase of ln-transformed RANTES 0.64, 95% CI 0.45-0.91, p=0.013) was also significantly associated with the composite of non-culprit lesion related and indeterminate death or ACS only. After adjustment

for clinical characteristics in multivariable analysis, RANTES remained independently predictive for non-culprit lesion related and indeterminate MACE (HR per SD increase of ln-transformed RANTES 0.71, 95% CI 0.53-0.96, p=0.026) and for non-culprit lesion related and indeterminate death or ACS only (HR per SD increase of ln-transformed RANTES 0.64, 95% CI 0.44-0.94, p=0.022) (Table 5).

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

	Unadjusted model		Age and gender adjusted model		Age, gender and indication for catheterization adjusted model		Full model*	
	HR (95% CI)	*P*	*HR (95% CI)*	*P*	*HR (95% CI)*	*P*	*HR (95% CI)*	*P*
Major adverse cardiac events (primary endpoint)								
RANTES	0.67 (0.50-0.89)	0.005	0.72 (0.54-0.96)	0.024	0.71 (0.53-0.95)	0.023	0.71 (0.53-0.96)	0.026
Composite of death or acute coronary syndrome (secondary endpoint)								
RANTES	0.64 (0.45-0.91)	0.013	0.69 (0.48-0.99)	0.046	0.64 (0.44-0.93)	0.021	0.64 (0.44-0.94)	0.022

Hazard ratios are per standard deviation increase in ln-transformed biomarker concentration.
* Variables entered into the full model were age, gender, diabetes mellitus, hypertension, history of myocardial infarction and indication for coronary catheterization. RANTES indicates Regulated upon Activation Normal T cell Expressed and Secreted.

DISCUSSION

This study investigated the relations of circulating chemokine concentrations with extensiveness of coronary atherosclerosis, amount of necrotic core, the presence of TCFA lesions and occurrence of future major adverse cardiac events in patients who underwent coronary angiography for ACS or stable angina pectoris. To our best knowledge, this is the first study that correlates circulating chemokines with in-vivo measurements of coronary atherosclerosis using IVUS virtual histology. Higher plasma MCP-1, MIP-1α, and lower RANTES concentrations were all associated with higher coronary plaque burden and more advanced plaque phenotypes as determined by IVUS (figure 2). However, only RANTES was found to be independently predictive for the occurrence of MACE, particularly of death and ACS.

Circulating chemokines

Chemokines are small cytokines that have the ability to induce directed chemotaxis of nearby leukocytes. MCP-1, MIP-1α, MIP-1β and RANTES belong to the C-C motif chemokine ligand (CCL) family and are also known as CCL2, CCL3, CCL4 and CCL5, respectively. (5-6) Pathologic studies have shown that these chemokines are highly expressed in

atherosclerotic plaques.(15-17) Animal studies have shown that these chemokines are actively involved in atherogenesis and plaque destabilization.(5-6) Furthermore, several epidemiological studies have indicated that serum or plasma levels of MCP-1, MIP-1α, MIP-1β and RANTES may predict future cardiac events.(5) However, their clinical utility as biomarker for cardiovascular risk stratification remains unclear.(5-6) We sought to further elucidate the correlations of circulating chemokine concentrations with in-vivo measurements of extensiveness, phenotype and vulnerability of coronary atherosclerosis by using IVUS.

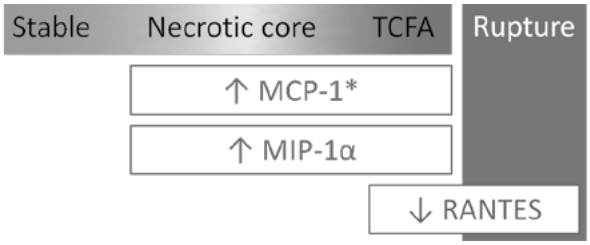

Figure 2.

Intravascular ultrasound

Grey-scale IVUS allows for in-vivo measurements of coronary plaque burden. Additionally, radiofrequency IVUS allows for differentiation of the composition of the atherosclerotic plaque and is therefore also known as IVUS virtual histology.(10) Necrotic core is often found in the more advanced and rupture-prone plaques.(18) The Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT) study has demonstrated that TCFA lesions as determined by IVUS are associated with MACE.(19) The strong and independent associations (adjusted hazard ratios ranging from 1.79 to 3.35) of IVUS-derived TCFA with MACE emphasize its biological importance.(18-20) However, there are several reasons why IVUS is currently not suitable for use as diagnostic and prognostic tool in the overall population of patients with coronary artery disease.(19) Its invasiveness is probably the most important limitation in this respect. Therefore, circulating biomarkers may have an important role in cardiovascular risk assessment.

RANTES

In our study, lower plasma RANTES concentrations were independently associated with adverse outcomes during 1 year of follow-up. Its association with acute cardiac events (death or ACS; HR 0.64) seemed to be even stronger than with all major adverse cardiac events (death, ACS or unplanned coronary revascularization; HR 0.71). This may indicate that RANTES is especially predictive for plaque rupture rather than plaque growth. Our finding

that low serum RANTES concentrations, rather than high, are associated with adverse coronary events may seem counterintuitive, since animal studies have shown that RANTES and its receptor are actively involved in atherogenesis and that RANTES was found to be highly expressed within atheromous lesions.(6,21) However, the inverse associations of RANTES may be explained by increased deposition of RANTES on the vascular endothelium, resulting in lower free circulating serum concentrations.(22-23) The inverse associations of RANTES are also consistent with observations from previous studies. A large case-control study reported that serum RANTES levels were lower in coronary heart disease patients compared with age- and gender-matched controls.(22) Another study reported that low plasma RANTES levels were independently associated with cardiac mortality in 389 male patients who underwent coronary angiography.(23) Such an association was not found in a population-based case-cohort study that included 363 individuals with incident coronary events and 1908 non-cases.(24)

MCP-1

We found that higher plasma MCP-1 concentrations were associated with higher coronary plaque burden in patients who were admitted with stable angina pectoris. These findings are in line with a previous study that measured MCP-1 concentrations in blood from the coronary sinus and found that these levels were associated with the extent of coronary atherosclerosis as assessed on the coronary angiogram.(25) Although we observed that high MCP-1 concentrations were associated with a more advanced plaque phenotype (i.e. higher necrotic core fraction) and with the presence of TCFA lesions, MCP-1 was not predictive for future events. Previous epidemiological studies have shown that the ability of MCP-1 to predict subclinical coronary artery disease is somewhat disappointing, but that MCP-1 may have some value in predicting cardiovascular events in patients with overt coronary artery disease.(5) For example, a previous study found that MCP-1 was independently associated with the composite of death or myocardial infarction in a large cohort of 4244 patients with ACS.(26) This study also demonstrated that high MCP-1 values at 4 months after the initial ACS were still predictive for long-term mortality afterwards. A major difference with our study is that both culprit lesion related and non-culprit lesion related events were included in their study endpoints, while definite culprit lesion related events were excluded from our study endpoints. Furthermore, we may have lacked statistical power to detect the previously reported association.

MIP-1α

MIP-1α has been studied less extensively. We found that MIP-1α was associated with coronary plaque burden, necrotic core fraction and with the presence of large TCFA lesions. However, we did not observe a correlation between MIP-1α concentration and occurrence

of MACE. Another study, however, found that MIP-1α was predictive for recurrent ACS in a relatively small cohort of 54 patients with unstable angina pectoris.(27) Further research is required to elucidate the role of MIP-1α in patients with coronary artery disease.

CONCLUSIONS

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

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4.3|

Acute phase proteins, VH-IVUS derived extent and composition of coronary atherosclerosis, and major adverse cardiac events

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Submitted

ABSTRACT

Introduction: We examined whether the acute phase proteins (APPs) Alpha-1-Antitrypsin, Alpha-2-Macroglobulin Complement C3, ferritin, haptoglobin, and Plasminogen Activator Inhibitor 1 (PAI-1) are associated with cardiovascular outcome, as well as with the extent and composition of coronary atherosclerosis as determined by intravascular ultrasound (IVUS) virtual histology (VH).

Methods: In 2008-2011, IVUS(-VH) imaging of a non-culprit coronary artery was performed in 581 patients from the ATHEROREMO-IVUS study undergoing coronary angiography for acute coronary syndrome (ACS) (n=318) or stable angina pectoris (SAP) (n=263). Coronary atherosclerotic plaque volume, composition (fibrous, fibro-fatty, dense calcium and necrotic core) and vulnerability (VH-derived thin-cap fibroatheroma (TCFA) lesions) were assessed. Major adverse cardiac events (MACE; all-cause mortality, ACS or unplanned coronary revascularization) were assessed during 1-year follow-up. We applied linear, logistic and Cox regression.

Results: Mean age was 61.5 ± 11.4 years and 75.4% were men. Higher ferritin was associated with higher coronary plaque volume (beta [95%CI]: 0.19 [0.07-0.31] percent atheroma volume, for the highest vs the lowest tertile of ferritin; p for linear association =0.013. Higher PAI-1 was associated with higher rates of all-cause mortality or ACS (hazard ratio [95%CI]: 2.98 [1.10-8.06], for the highest vs the lowest tertile of PAI-1. No clear-cut associations could be demonstrated between APPs and composition of atherosclerosis or plaque vulnerability.

Conclusions: Higher circulating ferritin was associated with higher coronary plaque volume, and higher PAI-1 was associated with higher incidence of all-cause mortality or ACS. None of the APPs displayed consistent associations with composition of atherosclerosis or plaque vulnerability.

INTRODUCTION

Chronic inflammation of the arterial wall plays an important role in the development of atherosclerosis, and it regulates aspects of plaque biology that trigger the thrombotic complications of atherosclerosis (1). Inflammation is commonly characterized by increased plasma concentrations of acute phase proteins (APPs). Several studies have demonstrated the ability of the APP C-reactive protein (CRP) to predict adverse coronary events in patients with stable and unstable coronary artery disease (CAD)(2). In order to further explore the nature of the association of CRP with coronary atherosclerosis, we have previously examined its relation with intravascular ultrasound (IVUS) virtual histology (VH) derived measures of coronary atherosclerosis(3). The results showed that higher CRP levels were associated with a higher coronary plaque burden, but that they were not associated with plaque vulnerability, which was defined as the presence of IVUS-VH- derived thin-cap fibroatheroma (VH-TCFA) lesions.The relation between other APPs and cardiovascular disease has generally been examined to a much smaller extent, and in particular studies on APPs in relation to an in-vivo assessment of the extent and composition of coronary atherosclerosis are lacking (4, 5).

APP's including Alpha-1-Antitrypsin (AAT), Alpha-2-Macroglobulin (α2M), Complement C3 (C3), ferritin, haptoglobin, and Plasminogen Activator Inhibitor 1 (PAI-1), are produced by the liver in response to circulating cytokines(6) . APPs contribute to the restoration of homeostasis by neutralizing inflammatory agents, help to minimize the extent of local tissue damage and participate in tissue repair and regeneration(6). Circulating levels of APPs may potentially be useful for risk stratification of patients with known CAD, and studies on their relation with the extent and composition of coronary atherosclerosis may provide further pathophysiological insights with regard to the mechanisms of progression and destabilization of atherosclerotic plaques.

Therefore, the purpose of this study is to examine the associations of AAT, α2M, C3, ferritin, haptoglobin, and PAI-1 with the extent and composition of coronary atherosclerosis as determined in-vivo by IVUS-VH, in patients undergoing coronary angiography. Furthermore, the prognostic value of the APPs for major adverse cardiac outcome during 1 year follow-up in these patients is investigated.

MATERIAL AND METHODS

Study population

The design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study has been described elsewhere (7). In brief, 581 patients who underwent diagnostic coronary

angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS) or stable angina pectoris (SAP) have been included from November 2008 to January 2011 in the Erasmus MC, Rotterdam, the Netherlands. Intravascular ultrasound (IVUS) of a non-culprit coronary artery was performed subsequent to angiography. The ATHEROREMO-IVUS study has been approved by the human research ethics committee of Erasmus MC, Rotterdam, the Netherlands. Written informed consent was obtained from all included patients and the study protocol conforms to the ethical guidelines of the Declaration of Helsinki.

Biomarkers

Blood samples were drawn from the arterial sheath prior to the diagnostic coronary angiography or PCI procedure, and were available in 570 patients for the current study. The blood samples were transported to the clinical laboratory of the Erasmus MC for further processing and storage at a temperature of -80 ^{0}C within two hours after blood collection. CRP was measured in the clinical laboratory of the Erasmus MC in serum samples using a 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^{o}C) to Myriad RBM, Austin, Texas, USA, where the concentrations of AAT, α2M, C3, ferritin, haptoglobin, and PAI-1, were measured using a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, Texas, USA). While ferritin, haptoglobin, and PAI-1 were determined in the full cohort of 570 patients, AAT, α2M, and C3, were determined in a random subset of 473 patients. This difference in numbers resulted from batch-wise handling of the samples in combination with an update of the composition of the multiplex assay by the manufacturer in-between two batches.

Intravascular ultrasound

Following the standard coronary angiography or PCI procedure, IVUS data were acquired in a non-culprit coronary artery without significant coronary disease requiring balloon angioplasty or stent treatment. The order of preference for selection of the non-culprit vessel was: 1. left anterior descending (LAD) artery; 2. Right coronary artery (RCA); 3. Left circumflex (LCX) artery. All IVUS data were acquired with the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, CA, USA) using a Vulcano Eagle Eye Gold IVUS catheter (20 MHz). An automatic pullback system was used with a standard pull back speed of 0.5 mm per second. The IVUS images were analyzed offline by an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands) that had no knowledge of clinical data. The IVUS radiofrequency analyses, also known as IVUS virtual histology (IVUS-VH), were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, CA, USA) software. The

external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40 mm). Extent and phenotype of the atherosclerotic plaque were assessed.

Plaque volume was defined as the percent of the volume of the external elastic membrane occupied by atheroma, i.e. percent atheroma volume(8). Plaque volume was normalized for the length of the imaged segment. Plaque burden was defined as the plaque and media cross-sectional area divided by the external elastic membrane cross-sectional area and is presented as a percentage. A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least 3 consecutive frames. Using IVUS-VH, the composition of the atherosclerotic plaque was characterized into 4 different types: fibrous, fibro-fatty, dense calcium and necrotic core (9). A IVUS-VH-derived thin-cap fibroatheroma (VH-TCFA) lesion was defined as a lesion with presence of > 10% confluent necrotic core in direct contact with the lumen.

Clinical study endpoints

In this study, follow-up lasted up to 1 year after angiography. Post-discharge survival status was obtained from municipal civil registries. Post-discharge rehospitalizations were prospectively assessed. Questionnaires focusing on the occurrence of major adverse cardiac events (MACE) were sent to all living patients. Subsequently, hospital discharge letters were obtained and treating physicians and institutions were contacted for additional information whenever necessary. The primary endpoint was the occurrence of MACE, defined as the composite of all-cause mortality, ACS or unplanned coronary revascularization. The secondary endpoint was the composite of all-cause mortality or ACS. ACS was defined as the clinical diagnosis of ST segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris in accordance with the guidelines of the European Society of Cardiology.(10, 11) Unplanned coronary revascularization was defined as unplanned repeat PCI or coronary artery bypass grafting (CABG). The endpoints were adjudicated by a clinical event committee that had no knowledge of biomarkers and IVUS data.

Statistical analysis

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

In further analyses, biomarker concentrations were examined both as continuous and as categorical variables (the latter by dividing the variables into tertiles). Biomarkers with a non-normal distribution were ln-transformed or were transformed by using the square root.

First, we examined associations of biomarker concentrations with the extent of atherosclerosis according to IVUS. We applied linear regression analyses with biomarker concentrations as the independent variable (transformed or categorized when appropriate) and, consecutively, plaque volume and plaque burden in the imaged coronary segment as the dependent variable. The results are presented as βs (per unit increase in transformed biomarker concentration or per category of biomarker concentration) with 95% confidence intervals (95% CIs).

Subsequently, we examined the associations between biomarker concentrations and 4 types of atherosclerosis composition (fibrous, fibrofatty, necrotic core, and dense calcium), each expressed in percentages. The results are again presented as βs. We also examined the associations between biomarker concentrations and the presence of VH-TCFA lesions by using logistic regression analyses with biomarker concentrations as the independent variable. The results are presented as odds ratios (ORs) per unit increase in transformed biomarker concentration or per category of biomarker concentration, with 95% CIs.

Moreover, we examined associations of biomarker concentrations with MACE and with the composite of all-cause mortality or ACS, during 1 year follow-up. Patients lost to follow-up were considered at risk until the date of last contact, at which time-point they were censored. We used Cox proportional hazard regression analyses with biomarker concentration as the independent variable. The results are presented as hazard ratios (HRs) per unit increase in ln-transformed biomarker concentration or per category of biomarker concentration, with 95% CIs.

All above-described analyses were performed univariably. Subsequently, we adjusted for age, gender, indication for coronary angiography, diabetes, hypertension and CRP. Additionally, to further examine possible effect modification by indication for baseline coronary angiography, we repeated the analyses separately in patients with acute coronary syndrome and in patients with stable angina pectoris.

All data were analyzed with SPSS software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Table 1. Baseline characteristics

	Total (n=570)	ACS patients (n=309)	SAP patients (n=261)
Patient characteristics			
Age, years, mean±standard deviation	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Men, 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, 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 renal insufficiency, n (%)	32 (5.6)	13 (4.2)	19 (7.3)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
Procedural characteristics			
Indication for coronary angiography			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	0 (0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0)
Unstable angina pectoris, n(%)	150 (26.3)	150 (48.5)	0 (0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0)	261 (100)
Coronary artery disease			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.4)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
Serum biomarker concentrations			
C-reactive protein (mg/L), median (IQR)	2.1 [0.8-5.3]	2.8 [1.1-7.0]	1.5 [0.6-3.1]
Alpha-1-Antitrypsin (mg/mL)*, median (IQR)	1.40 [1.20-1.70]	1.40 [1.20-1.70]	1.40 [1.20-1.60]
Alpha-2-Macroglobulin (mg/mL)*, median (IQR)	1.50 [1.40-1.80]	1.50 [1.30-1.80]	1.60 [1.40-1.80]
Complement C3 (mg/mL)*, median (IQR)	0.90 [0.78-1.10]	0.90 [0.78-1.10]	0.92 [0.79-1.00]
Ferritin (mg/mL)#, median (IQR)	173.00 [94.50-282.50]	191.00 [102.25-319.00]	144.00 [82.00-242.50]
Haptoglobin (mg/mL)#, median (IQR)	1.40 [0.91-2.01]	1.50 [0.98-2.20]	1.26 [0.85-1.90]
Plasminogen Activator Inhibitor 1 (ng/mL)+, median (IQR)	35.00 [23.00-53.00]	38.00 [27.00-61.50]	31.00 [21.00-46.00]

AAT, α2M, and C3: total n= 473, ACS n=309, SAP n= 261
*Measurable in all 473 patients
+Measurable in all 570 patients
#Measurable in >99% of 570 patients, too low to detect in <1%

RESULTS

Baseline characteristics

Baseline characteristics are summarized in Table 1. Mean age was 61.5 ± 11.4 years and 75% were men. Coronary angiography or PCI was performed for several indications: 159 (28%) patients had an acute myocardial infarction, 150 (26%) patients had unstable angina pectoris and 261 (46%) patients had stable angina pectoris. The median length of the imaged coronary segment was 44.1 [33.7-55.4] mm. C3 was the only biomarker with a normal distribution. AAT, α2M, ferritin, haptoglobin and PAI-1 concentrations were not normally distributed; haptoglobin was square root-transformed for further analyses, and the remaining biomarkers were ln-tranformed.

Biomarkers and extent of atherosclerosis

The results of the analyses for (ln-transformed) normalized plaque volume normalized for the length of the segment are shown in table 2. Higher ferritin levels were associated with higher coronary plaque volume (β [95%CI]: 0.19 [0.07-0.31], for the highest vs the lowest tertile of ferritin, and β [95%CI]: 0.14 [0.02-0.27], for the middle vs the lowest tertile of ferritin; p for linear association =0.01). After multivariable adjustment, only the association for the highest vs the lowest tertile of ferritin persisted (Table 2). In a post-hoc analysis, we performed the multivariable adjustment without adding CRP to the model (thus, only adjusting for age, gender, indication, diabetes and hypertension). The association was independent of these clinical covariates (β [95%CI]: 0.17 [0.05-0.30], for the highest vs the lowest tertile of ferritin, and β [95%CI]: 0.13 [0.006-0.25], for the middle vs the lowest tertile of ferritin; p for linear association =0.045) No associations were present between any of the other biomarkers and coronary plaque volume.

Higher ferritin was associated with higher plaque burden (β [95%CI]: 1.22 [0.07-2.38], for the highest vs the lowest tertile of ferritin). However, p for linear association was not statistically significant (p=0.24).

Biomarkers and composition of atherosclerosis

The results of the analyses for composition of atherosclerosis are shown in Figure 1. Higher α2M levels were associated with a lower percentage of fibrous tissue (β [95%CI]: -4.60 [-7.10 - -2.10], for the highest vs the lowest tertile of α2M, and β [95%CI]: -2.65 [-5.02- -0.28], for the middle vs the lowest tertile of α2M; p for linear association =0.005). Furthermore, a higher α2M level was associated with a higher percentage of dense calcium tissue (β [95%CI]: 0.49 [0.23-0.375], for the highest vs the lowest tertile of α2M, and β [95%CI]: 0.30 [0.05-0.55], for the middle vs the lowest tertile of α2M; p for linear association =0.003). After multivariable adjustment, the associations for the highest tertiles of α2M remained

significant, but the trends lost significance. No associations were present between α2M and the remaining atherosclerosis components.

Table 2. Association of AAT, α2M, C3, ferritin, haptoglobin, and PAI-1 with segment plaque volume

Segmental plaque volume	**Unadjusted model**		**Multivariable model***	
	beta (95%CI)	P	beta (95%CI)	P
AAT				
Tertile 1	(reference)		(reference)	
Tertile 2	0.11 (-0.01-0.23)	0.08	0.08 (-0.04-0.21)	0.19
Tertile 3	0.09 (-0.04-0.22)	0.19	0.10 (-0.04-0.24)	0.15
Ln (AAT)	0.07 (-0.13-0.27)	0.50	0.03 (-0.19-0.24)	0.82
α2M				
Tertile 1	(reference)		(reference)	
Tertile 2	0.01 (-0.11-0.14)	0.83	-0.003 (-0.13-0.12)	0.97
Tertile 3	0.06 (-0.07-0.18)	0.40	0.08 (-0.05-0.22)	0.23
Ln (α2M)	-0.10 (-0.35-0.14)	0.40	-0.09 (-0.35-0.16)	0.47
C3				
Tertile 1	(reference)		(reference)	
Tertile 2	0.02 (-0.11-0.15)	0.76	0.00 (-0.13-0.13)	1.00
Tertile 3	-0.05 (-0.18-0.07)	0.39	-0.03 (-0.16-0.10)	0.66
C3	-0.16 (-0.41-0.10)	0.22	-0.15 (-0.41-0.12)	0.27
Ferritin				
Tertile 1	(reference)		(reference)	
Tertile 2	0.14 (0.02-0.27)	0.023	0.12 (-0.01-0.24)	0.061
Tertile 3	0.19 (0.07-0.31)	0.003	0.16 (0.03-0.29)	0.014
Ln (Ferritin)	0.07 (0.02-0.13)	0.013	0.05 (-0.01-0.11)	0.077
Haptoglobin				
Tertile 1	(reference)		(reference)	
Tertile 2	-0.04 (-0.16-0.09)	0.58	-0.02 (-0.15-0.11)	0.76
Tertile 3	-0.004 (-0.13-0.12)	0.95	0.003 (-0.12-0.13)	0.95
square root (haptoglobin)	-0.003 (-0.13-0.13)	0.97	-0.01 (-0.15-0.13)	0.89
PAI-1				
Tertile 1	(reference)		(reference)	
Tertile 2	0.09 (-0.04-0.21)	0.17	0.09 (-0.03-0.21)	0.15
Tertile 3	-0.03 (-0.15-0.10)	0.65	0.03 (-0.10-0.15)	0.67
Ln (PAI-1)	-0.02 (-0.10-0.06)	0.70	0.03 (-0.06-0.11)	0.55

*adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP

Higher ferritin levels were associated with a higher percentage of fibrofatty tissue (β [95%CI]: 0.25 [0.05-0.46], for the highest vs the lowest tertile of ferritin; p for linear association=0.015) (Figure 1). The trend disappeared after multivariable adjustment. Ferritin was not associated with other VH-IVUS-defined components.

Remaining biomarkers did not display any associations with the 4 components of atherosclerosis. Furthermore, associations with individual components of atherosclerosis were not reflected by associations with IVUS-VH-derived TCFA: none of the biomarkers were associated with IVUS-VH derived TCFA (Table 3).

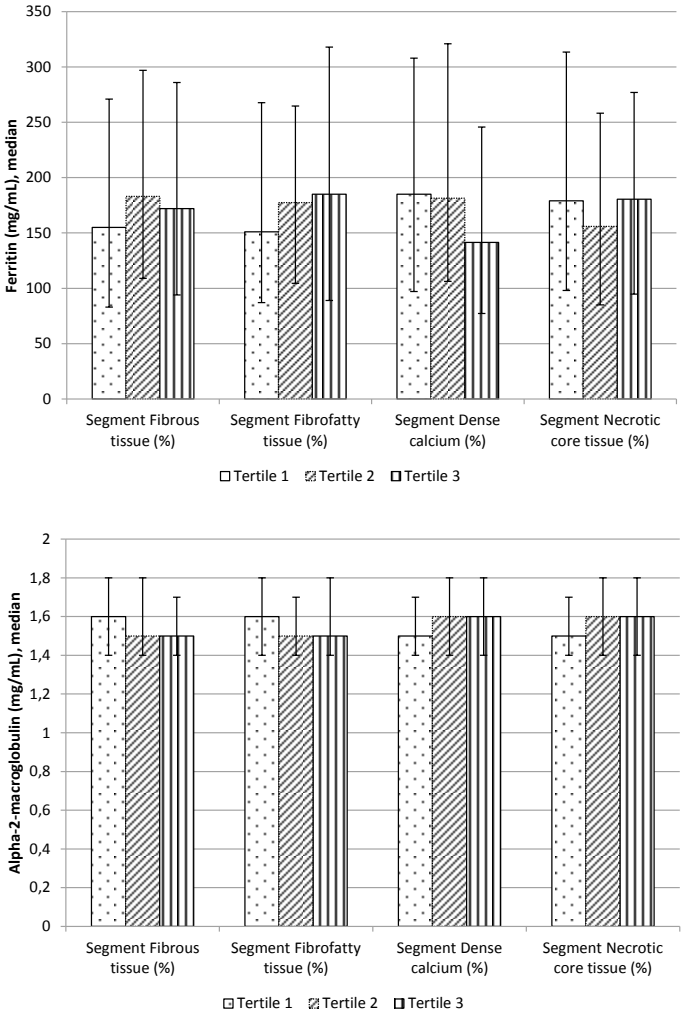

Figure 1. Alpha-2-macroglobulin and ferritin and composition of atherosclerosis

Table 3. Association of AAT, α2M, C3, ferritin, haptoglobin, and PAI-1 with presence of IVUS-VH derived TCFA lesions

VH-TCFA	Unadjusted model		Multivariable model*	
	OR (95%CI)	P	OR (95%CI)	P
AAT				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.35 (0.85-2.15)	0.20	1.24 (0.78-1.99)	0.37
Tertile 3	1.19 (0.75-1.91)	0.46	1.09 (0.66-1.77)	0.74
LN (AAT)	1.13 (0.55-2.33)	0.74	0.94 (0.42-2.12)	0.89
α2M				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.09 (0.71-1.70)	0.69	1.16 (0.74-1.81)	0.53
Tertile 3	1.07 (0.67-1.69)	0.79	1.16 (0.71-1.89)	0.55
LN (α2M)	1.00 (0.41-2.41)	0.99	1.22 (0.47-3.13)	0.68
C3				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.05 (0.67-1.66)	0.83	1.02 (0.64-1.63)	0.92
Tertile 3	0.99 (0.63-1.56)	0.97	0.95 (0.60-1.52)	0.84
C3	0.72 (0.29-1.79)	0.48	0.58 (0.22-1.56)	0.28
Ferritin				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.09 (0.72-1.63)	0.69	1.07 (0.70-1.62)	0.77
Tertile 3	1.04 (0.69-1.57)	0.85	0.96 (0.63-1.48)	0.87
LN (Ferritin)	1.03 (0.86-1.25)	0.73	1.01 (0.83-1.23)	0.92
Haptoglobin				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.07 (0.71-1.60)	0.76	1.05 (0.69-1.58)	0.84
Tertile 3	0.81 (0.53-1.22)	0.31	0.75 (0.48-1.16)	0.19
Square root (haptoglobin)	0.81 (0.52-1.25)	0.34	0.72 (0.45-1.18)	0.19
PAI-1				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.05 (0.70-1.58)	0.81	0.98 (0.65-1.49)	0.93
Tertile 3	1.00 (0.66-1.49)	0.98	0.94 (0.62-1.43)	0.77
LN (PAI-1)	1.05 (0.80-1.37)	0.73	1.01 (0.76-1.33)	0.96

*adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP

Biomarkers and outcome

Vital status was acquired for 569 (99.8%) patients. Response rate of the questionnaires that were sent to all living patients was 92.3%. After 1 year of follow-up, 56 patients experienced at least 1 MACE (%). No significant associations were found between the APPs and MACE. A total of 30 cases of all-cause mortality or ACS occurred. Hazard ratios for the occurrence of the composite of all-cause mortality or ACS are shown in Table 4. Higher PAI-1 was associated with higher event rates (HR[95%CI]: 2.98 [1.10-8.06], for the highest vs the lowest tertile of PAI-1, and HR[95%CI]: 3.46 [1.30-9.22], for the middle vs the lowest tertile of PAI-1). After multivariable adjustment, associations remained significant. No associations were present between any of the other biomarkers and all-cause mortality or ACS.

Patients with ACS versus stable angina pectoris

To further investigate the possibility of effect modification by baseline indication for coronary angiography, we repeated all analyses separately in patients with ACS and patients with SAP. The association between ferritin and higher percentage of fibrofatty tissue was present in patients with ACS (β [95%CI]: 0.56 (0.27-0.86) for the highest vs the lowest tertile of ferritin, p for linear association < 0.001), but was not present in patients with SAP. Remaining estimates remained materially the same in both subgroups, although statistical significance disappeared on some occasions because of lower statistical power in the subgroups.

DISCUSSION

In this paper, we examined whether circulating APP concentrations are associated with the extent and composition of coronary atherosclerosis, as determined by (VH)-IVUS, in patients undergoing coronary angiography. We also investigated whether these APPs have prognostic value for clinical cardiovascular outcome. Higher concentrations of ferritin were associated with higher coronary plaque volume as well as a higher percentage of fibrofatty tissue in the coronary atherosclerotic plaque, the latter only in patients with acute coronary syndrome at baseline. Furthermore, higher concentrations of α2M were associated with lower percentage of fibrous tissue and higher percentage of dense calcium tissue in the coronary atherosclerotic plaque. In spite of these associations with individual components of atherosclerosis, none of the biomarkers were associated with presence of IVUS-VH derived TCFA lesions. Higher concentrations of PAI-1 were associated with the composite endpoint of all-cause mortality or ACS. No significant associations were found between the other APPs and the clinical endpoints.

Table 4. Association of AAT, α2M, C3, ferritin, haptoglobin, and PAI-1 with combination mortality and ACS

All-cause mortality or ACS	Unadjusted model		Multivariable model*		Multivariable model#	
	HR (95%CI)	P	HR (95%CI)	P	HR (95%CI)	P
AAT						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.37 (0.10-1.38)	0.14	0.18 (0.03-1.07)	0.059	0.30 (0.07-1.20)	0.089
Tertile 3	0.33 (0.08-1.267)	0.11	0.18 (0.03-0.97)	0.046	0.556 (0.13-2.36)	0.43
Ln (AAT)	0.31 (0.06-1.72)	0.18	0.30 (0.06-1.59)	0.16	0.64 (0.07-5.66)	0.69
α2M						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.35 (0.52-3.51)	0.54	1.45 (0.54-3.91)	0.47	1.18 (0.32-4.41)	0.81
Tertile 3	1.34 (0.46-3.87)	0.59	1.35 (0.43-4.28)	0.61	1.44 (0.33-6.24)	0.63
Ln (α2M)	2.99 (0.38-23.62)	0.30	3.26 (0.35-30.86)	0.30	3.02 (0.14-66.71)	0.48
C3						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.99 (0.35-2.81)	0.99	1.12 (0.35-3.54)	0.85	0.73 (0.23-2.32)	0.59
Tertile 3	1.77 (0.60-5.23)	0.30	2.41 (0.51-11.32)	0.27	2.11 (0.68-6.57)	0.20
C3	1.60(0.11-23.40)	0.73	1.08 (0.02-61.46)	0.97	2.94 (0.18-48.21)	0.45
Ferritin						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.80 (0.30-2.10)	0.65	1.03 (0.35-2.98)	0.96	0.56 (0.20-1.57)	0.27
Tertile 3	1.54 (0.62-3.84)	0.36	2.21 (0.71-6.93)	0.17	1.34 (0.53-3.43)	0.54
Ln (Ferritin)	1.04 (0.71-1.53)	0.83	1.12 (0.74-1.70)	0.59	0.98 (0.64-1.49)	0.91
Haptoglobin						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.37 (0.14-1.00)	0.049	0.33 (0.12-0.94)	0.037	0.434 (0.16-1.20)	0.12
Tertile 3	0.49 (0.20-1.18)	0.11	0.40 (0.14-1.10)	0.074	0.89 (0.30-2.66)	0.84
square root (haptoglobin)	0.77 (0.31-1.87)	0.56	0.64 (0.23-1.76)	0.39	1.04 (0.40-2.76)	0.93
PAI-1						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	3.46 (1.30-9.22)	0.013	3.57 (1.32-9.66)	0.012	3.64 (1.27-10.43)	0.016
Tertile 3	2.98 (1.10-8.06)	0.032	4.12 (1.04-16.41)	0.045	3.51 (1.26-9.74)	0.016
Ln (PAI-1)	1.52 (0.94-2.46)	0.091	2.02 (0.99-4.09)	0.052	1.74 (1.01-2.99)	0.047

*adjusted for age, gender and indication for coronary angiography
#additionally adjusted for diabetes mellitus, hypertension and CRP

Ferritin plays a fundamental role in the storage of intracellular iron. Consequently, this marker is widely used in diagnosing and monitoring of diseases associated with iron overload or iron deficiency(12). Tran et al. (13) showed, in vivo, that serum ferritin concentration increases in response to the inflammatory cytokines interleukin-1 and TNF-α, suggesting that these cytokines upregulate ferritin and its secretion. Elevated serum concentrations of ferritin are seen in several inflammatory conditions like chronic kidney disease, acute infection, and malignancy(13). Recently, Sung et al. (14) found a significant association between elevated levels of ferritin and the presence of coronary artery calcium, a marker of preclinical atherosclerosis. This association was independent of cardiovascular risk factors, iron-binding capacity (transferrin), and low-grade inflammation. However, in general, clinical studies have given contradictory results regarding the ability of ferritin to predict cardiovascular events (15-18). In our study, higher ferritin levels were clearly associated with higher coronary atherosclerotic plaque volume, but their association with higher plaque burden was less apparent. This seeming discrepancy may be due to the fact that plaque burden is not a direct measure of three dimensional plaque volume, but rather a two dimensional measure that also accounts for arterial wall remodelling.

Furthermore, higher ferritin levels were associated with higher percentage of fibrofatty tissue in coronary atherosclerotic plaque, the latter only in patients with acute coronary syndrome at baseline. However, no association was found with VH-TCFA lesions, nor with clinical cardiovascular events. Since serum concentrations of ferritin may vary in a wide range of conditions (19), the interpretation of ferritin level may be complicated and additional research is needed to confirm its potential association with extent of coronary atherosclerosis.

α2M is particularly known as an APP that can bind a large array of ligands and remove them from blood circulation to protect the body from wide disturbances (20). Its primary function is the inhibition of fibrinolysis which, under normal physiological conditions, contributes to stabilization of vascular thrombus(21). Furthermore, this protein is fundamental for enabling smooth muscle cells to attach, migrate and survive in fibrin (22), which suggests that this marker is involved in the mechanism of vascular stenosis. Clinical studies have shown that levels of α2M are significantly influenced by nephrotic syndrome, diabetes mellitus, and chronic liver disease (23). Moreover, the cardiac isoform of A2macro seems to be useful in diagnosing cardiac events in patients with diabetes (24, 25) and in HIV patients (26, 27). In the current study, we found a significant association of elevated α2M levels with presence of lower percentage of fibrous tissue and higher percentage dense calcium tissue in the atherosclerotic plaque. However, these associations with plaque composition could not be further translated into associations with number of VH-TCFA lesions.

PAI-1 was initially described as a protein that controls the plasminogen activation system, leading to inhibition of endogenous fibrinolysis and shifting the dynamic balance towards

fibrin generation(28). Circulating concentrations of PAI-1 are elevated in both the elderly (29) and in the presence of several clinical characteristics, including hypertriglyceridemia (29), obesity, insulin resistance, decreased immune responses, and increased inflammation(30). Although PAI-1 may contribute to the development of the atherosclerotic plaque by stabilizing the fibrin matrix, this protein is also involved in, for example, vascular smooth muscle migration, and activation of specific matrix metalloproteases (MMPs) that diminish plaque rupture(28, 31). This phenomenon is also known as the 'PAI-1 paradox'(28). Nevertheless, most epidemiological studies have showed that PAI-1 is a risk factor for the development (32, 33) and recurrence of cardiovascular disease(34, 35). Furthermore, a recent meta-analysis (36) has suggested that the presence of the 4G/5G gene polymorphism of PAI-1, resulting in increased PAI-1 levels, is associated with increased risk of MI. Our findings, demonstrating that higher concentrations of PAI-1 are associated with higher acute cardiac event rates, are in line with these prior studies.

Previous studies on AAT deficiency (37, 38) and complement factor C3 (39) and cardiovascular disease have rendered contradictory results. A recent study on common haptoglobin variants showed that these variants modify the inflammatory response to intraplaque hemorrhage and increase the risk of major cardiovascular events(40). In particular, the hp2 allele of haptoglobin was associated with such events. In the current study, we could not demonstrate any associations of AAT, C3 and haptoglobin with coronary plaque characteristics or clinical events.

Some aspects of this study warrant consideration(7). IVUS-VH imaging was performed in a prespecified single target segment of a single non-culprit coronary artery, based on the assumption that such a non-stenotic segment would adequately reflect coronary wall pathophysiology of the larger coronary tree(8). Although this assumption may be debated, previous studies evaluating IVUS have demonstrated that the coronary wall of comparable non-culprit, non-stenotic segments of a single vessel does, in fact, reflect larger coronary disease burden and is associated with subsequent cardiac events (41, 42). Furthermore, it is important to note that IVUS is formally not capable of detecting TCFA lesions (7, 8, 43), because the spatial resolution of IVUS is insufficient for thin cap detection (8). Nonetheless, a concept of IVUS-VH derived TCFAs has been postulated for plaques with a plaque burden ≥ 40% and a confluent necrotic core ≥ 10% in direct contact with the lumen in at least three IVUS-VH frames (8). Notably, we have recently demonstrated that such IVUS-VH derived TCFA lesions are strongly and independently predictive of the occurrence of MACE within the current study population(42).

CONCLUSIONS

In this population of patients undergoing coronary angiography for ACS or SAP, higher circulating ferritin was associated with higher coronary plaque volume, and higher PAI-1 was associated with higher incidence of all-cause mortality or ACS during 1-year follow-up. Of the APPs we investigated, no clear-cut associations could be demonstrated with composition of coronary atherosclerosis or with plaque vulnerability, as assessed by IVUS-VH. Further research, using various intravascular imaging modalities, is warranted to provide additional insights into potential mechanisms through which APPs may affect composition of atherosclerosis.

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51

Smoking and coronary atherosclerosis

15

Smoking and coronary atherosclerosis

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Association of smoking with VH-IVUS derived extent and composition of coronary atherosclerosis

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Submitted

ABSTRACT

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

Methods and results: Between 2008-2011, (VH-)IVUS of a non-culprit coronary artery was performed in 581 patients undergoing coronary angiography. To account for differences in baseline characteristics, current smokers were matched to never smokers by age, gender and indication for catheterization, resulting in 280 patients available for further analysis. Coronary atherosclerotic plaque volume, burden and composition (fibrous, fibro-fatty, dense calcium and necrotic core) were assessed and high-risk lesions (VH-IVUS derived thin-cap fibroatheroma (TCFA), plaque burden ≥70%, minimal luminal area ≤4.0 mm^2) were identified. Cigarette smoking showed a tendency towards higher coronary plaque burden (mean±SD, 38.6±12.5% in current versus 36.4±11.0% in never smokers, p=0.080; and odds ratio (OR) of current smoking for plaque burden above versus below the median 1.69 (1.04-2.75), p=0.033). Fibrous tissue tended to be lower in current smokers (mean± SD, 57.7±10.5% versus 60.4±12.6%, p=0.050) and fibro-fatty tissue was higher in current smokers (median [IQR], 9.6[6.0-13.7]% versus 8.6[5.8-12.2]%, p=0.039). However, differences in percentage necrotic core and dense calcium could not be demonstrated. Also, no differences were found with regard to high-risk lesions.

Conclusions: A substantial association between smoking and degree of coronary atherosclerosis could not be demonstrated in patients undergoing coronary angiography. Although smoking was associated with higher fibro-fatty percentage, no associations could be demonstrated with percentage necrotic core, nor with VH-IVUS derived TCFA lesions.

INTRODUCTION

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

In line with the above, several pathophysiologic effects of cigarette smoke exposure on cardiovascular function have been described. Both active and passive cigarette smoke exposure have been shown to promote endothelial dysfunction, stimulate inflammatory processes at the vessel wall and enhance vascular prothrombotic effects (6, 7). Thus, ample fundamental research evidence is available demonstrating that cigarette smoking directly impacts multiple aspects of atherosclerosis. However, less is currently known about the associations of cigarette smoking with in vivo, macroscopic plaque composition and plaque vulnerability. Although coronary angiography enables evaluation of the unobstructed part of the lumen, it does not provide information on the structure of the arterial wall itself. Grayscale intravascular ultrasound (IVUS) also provides limited information on plaque characteristics. Histopathological studies examining coronary arteries suggest that smoking predisposes patients to coronary thrombosis rather than promoting the progression of atherosclerosis (8-11). However, given the post-mortem nature of these studies, inherent selection bias is present.

Virtual histology (VH)-IVUS of the coronary arteries allows spectral analysis of backscattered radiofrequency ultrasound signal and herewith enables in vivo analysis of the composition of coronary plaque as well as in vivo identification of VH-IVUS derived thin-cap fibroatheroma (TCFA) lesions (12). Until now, the association between smoking and in vivo coronary plaque composition has only been examined in two studies. The first (13, 14) applied VH-IVUS and examined several plaque components, but did not assess VH-IVUS derived TCFA. The second (15) used integrated backscatter IVUS, which is based on the same principle as VH-IVUS, but examined 30 patients only.

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

METHODS

Study population and baseline characteristics

This study was performed within the framework of the European collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study . The rationale and design of the ATHEROREMO-IVUS study have been described in detail elsewhere (16). In brief, 581 patients who underwent diagnostic coronary angiography or percutaneous coronary intervention (PCI) for ACS or stable CAD have been included in this study between 2008 and 2011 at the Erasmus MC, Rotterdam, the Netherlands. The ATHEROREMO-IVUS study has been approved by the human research ethics committee of the Erasmus MC. Written informed consent was obtained from all participants. The study is registered in ClinicalTrials.gov, number NCT01789411.

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

Intravascular ultrasound

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

The external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40 mm). Degree and phenotype of the atherosclerotic plaque were assessed. Plaque volume was defined as the percent of the volume of the external elastic membrane occupied by atheroma, i.e. percent atheroma volume. Plaque

burden was defined as plaque and media cross-sectional area divided by external elastic membrane cross-sectional area.

A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least 3 consecutive frames. Using VH-IVUS, the composition of the atherosclerotic plaques was characterized into 4 different tissue types: fibrous (FI), fibro-fatty (FF), dense calcium (DC) and necrotic core (NC) (17). These tissue type components were expressed as percentages of total plaque volume. Three types of high-risk lesions were identified: 1.VH-IVUS derived thin-cap fibroatheroma (TCFA) lesion, defined as a lesion with presence of >10% confluent necrotic core in direct contact with the lumen; 2. Lesion with large plaque burden, defined as a lesion with a plaque burden of ≥70%; 3. Stenotic lesion, defined as a lesion with a minimal luminal area of ≤4.0 mm^2 (17, 18). In addition, calcified VH-TCFA lesions were examined; these were defined as VH-TCFA lesions containing >10% of confluent dense calcium. Remodeling index was expressed as the external elastic membrane cross-sectional area at the site of minimal luminal area divided by the reference external elastic membrane cross-sectional area. The reference site was selected <10 mm proximal to the lesion. Positive remodeling (arterial expansion) was defined as a remodeling index of >1.05, and negative remodeling (arterial shrinkage) was defined as a remodeling index of <0.95.

STATISTICAL ANALYSIS

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

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

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

Subsequently, we performed conditional logistic regression to examine the associations between smoking status and high plaque burden (above versus below the median), as well as smoking status and the three types of high-risk lesions (VH-IVUS derived TCFA, lesion with plaque burden ≥70%, lesion with minimal luminal area ≤4.0 mm^2).
All data were analyzed with SPSS software (SPSS 20.0, IBM corp., Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.050 were considered statistically significant.

Table 1. Baseline clinical and procedural characteristics, before matching

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

Values are mean ± SD or n (%).
P-values were obtained by independent samples t-test or Chi-squared test, whichever was appropriate.

RESULTS

Baseline characteristics

Baseline clinical and procedural characteristics of the total patient population are presented in Table 1. Current (n=169) and never smokers (n=307) significantly differed at baseline. Current smokers, on average, were significantly younger than the never smokers (55.7±10.8 years vs. 64.4±10.8, p<0.001). Significantly more men were present among the current smokers (79.3% vs. 70.7%, p=0.041), and current smokers were less likely to have predisposing risk factors such as hypertension (p<0.001), dyslipidemia (p=0.030) and diabetes mellitus (p=0.038). Furthermore, the indication for coronary angiography or PCI significantly differed between the two groups. Current smokers more often underwent catheterization for ACS and less often for stable CAD compared to the never smokers (p<0.001).

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

Degree of coronary atherosclerosis

To assess differences in degree of atherosclerosis between current smokers and never smokers, plaque volume and plaque burden were examined in the coronary segments. Plaque volume (median (IQR)) was similar for current and never smokers (221.8[134.6-312.5]mm^3 versus 207.5[134.5-293.2]mm^3) (Table 3). On the other hand, with regard to plaque burden, there was a tendency towards slightly higher values in current smokers (Table 3). Plaque burden (mean±SD) was 38.6±12.5% in current smokers versus 36.4±11.0% in never smokers, p=0.080 (Figure 1). The odds ratio (OR) (95% confidence interval (CI)) of current smoking for plaque burden above the median versus below the median was 1.69 (1.04-2.75), p=0.033 (Table 4).

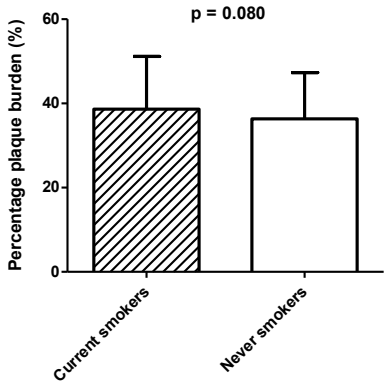

Figure 1. Difference in plaque burden between current and never smokers

Table 2. Baseline clinical and procedural characteristics, after matching

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

Values are mean ± SD or n (%).
P-values were obtained by paired samples t-test, McNemar test or Marginal Homogeneity, whichever was appropriate. MV=matching variable

The number of patients with ≥1 lesions did not significantly differ between current and never smokers (85.7% vs. 87.9%, p=0.72) (Table 3). Also, the odds ratio of having one or more lesions with plaque burden ≥70% was not significantly raised (OR (95% CI): 1.47 (0.76-2.83)), and neither was the odds ratio of having one or more lesions with a minimal luminal area of ≤4.0 mm^2 (Table 4).

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

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

Composition of coronary atherosclerosis

VH-IVUS segment and lesion characteristics of the matched smokers and never smokers are listed in Table 3. Percentage of fibrous tissue (% FI) volume in the examined coronary segment tended to be lower in current smokers (57.7±10.5% vs. 60.4±12.6%, p=0.050) and percentage of fibro-fatty tissue volume was higher in current smokers (9.6[6.0-13.7]% vs. 8.6[5.8-12.2]%, p=0.039) (Table 3, Figure 2). However, differences in percentage necrotic core (% NC) volume and dense calcium volume could not be demonstrated, and prevalence of ≥1 TCFA lesions was exactly the same in current and never smokers (both 40.7%, Table 3 and Table 4). Similarly, no differences could be demonstrated in prevalence of ≥1 calcified TCFA lesions (Table 3 and Table 4).

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

	Current smokers (n = 140)	**Never smokers** (n = 140)	**P-value**
(VH-)IVUS segment parameters			
Segment length, mm	45.4 ± 15.4	44.7 ± 13.2	0.67
Degree of atherosclerosis			
Plaque volume, mm^3	221.8 [134.6 - 312.5]	207.5 [134.5 - 293.2]	0.68
Plaque burden, %	38.6 ± 12.5	36.4 ± 11.0	0.080
Composition of atherosclerosis			
% FI volume	57.7 ± 10.5	60.4 ± 12.6	0.050
% FF volume	9.6 [6.0-13.7]	8.6 [5.8-12.2]	0.039
% NC volume	21.6 ± 8.0	20.8 ± 8.8	0.40
% DC volume	7.6 [4.7-13.9]	8.0 [4.3-13.3]	0.62
(VH-)IVUS lesion parameters			
≥1 Lesions, n (%)	120 (85.7)	123 (87.9)	0.72
Presence of high risk lesions, n (%)	91 (65.0)	83 (59.3)	0.39
High risk lesion type:			
Degree of atherosclerosis			
≥1 Lesion with plaque burden ≥70%, n (%)	32 (22.9)	27 (19.3)	0.32
≥1 Lesion with MLA ≤4.0mm^2, n (%)	43 (30.7)	42 (30.0)	0.67
Composition of atherosclerosis			
≥1 TCFA, n (%)	57 (40.7)	57 (40.7)	1.00
≥1 Calcified TCFA, n (%)	29 (20.7)	35 (25.0)	0.48

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

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

	OR (95% CI)	P-value
(VH-)IVUS segment parameters		
Plaque burden		
Below the median	1.00 (reference)	
Above the median	1.69 (1.04 – 2.75)	0.033
(VH-)IVUS lesion parameters		
≥1 Lesion with plaque burden ≥70%	1.47 (0.76 – 2.83)	0.25
≥1 Lesion with MLA ≤4.0mm^2	1.17 (0.67 – 2.05)	0.57
≥1 TCFA	1.00 (0.63 – 1.60)	1.00
≥1 Calcified TCFA	1.09 (0.62 – 1.92)	0.77

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

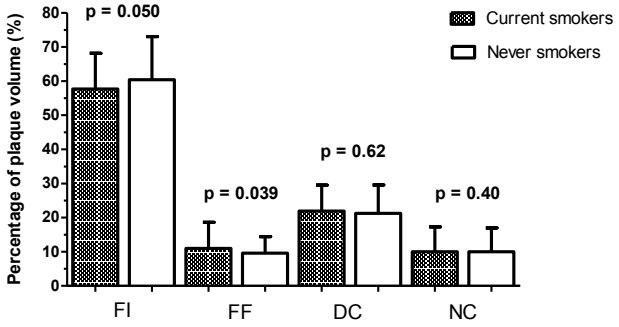

Figure 2. Difference in composition of coronary atherosclerosis between current and never smokers

DISCUSSION

This study investigated the associations of cigarette smoking with coronary atherosclerotic plaque burden, volume and composition as determined by (VH-)IVUS of a non-culprit section of a coronary artery in patients undergoing coronary angiography. Alhough cigarette smoking showed a tendency towards slightly higher coronary plaque burden, a substantial association between smoking and degree of coronary atherosclerosis could not be demonstrated. Furthermore, while cigarette smoking was associated with higher percentage of fibro-fatty plaque volume, no associations could be demonstrated with percentage necrotic core volume, nor with VH-IVUS derived TCFA lesions, suggesting that smoking has no major influence on plaque vulnerability.

Although several studies have examined the association between cigarette smoking and degree of coronary atherosclerosis as measured by IVUS (13-15, 19-21), so far only one

large study has applied virtual histology (VH-IVUS) to assess its association with composition of coronary atherosclerosis and plaque vulnerability. Philipp et al (14) examined 990 patients enrolled in a prospective, multicentre, global VH-IVUS registry, and found that smoking was not associated with a specific plaque composition in this study population of consecutive, nonselected patients, which is in line with our findings. However, they did not perform a lesion-based analysis, or a categorization into high-risk lesions such as TCFA, as we did. Missel et al (13) examined a subset of 473 male patients with de novo culprit coronary lesions from the same registry, and found that the NC/DC ratio was higher for smokers versus non-smokers in this subgroup. Other VH parameters were not significantly influenced by smoking. High NC/DC ratio was used as a measure of plaque vulnerability in the study by Missel et al. The association they demonstrated, was not present in the current study, in which we used TCFA as a measure of plaque vulnerability. When taking DC into further account by examining only calcified TCFA in the current study, results remained negative. The same applied to a post-hoc analysis examining NC/DC ratio (results not shown). An earlier, small study by Sano et al (15) examined plaque characteristics in 30 patients with stable angina pectoris, and did not find an association with smoking either. However, since only 6 patients smoked in this study, statistical power was very limited.

With regard to degree of coronary atherosclerosis as assessed by IVUS, previous studies have rendered contradicting results. Nicholls et al (20) demonstrated that smoking was a weak independent predictor of percent plaque volume on multivariable analysis in a population of 654 patients with a clinical indication for diagnostic coronary angiography from the REVERSAL trial. Furthermore, Von Birgelen et al found an association between smoking and progression of plaque plus media (P&M) cross-sectional area (CSA) in 56 patients with de novo, hemodynamically nonsignificant plaques. In contrast, Kahlon et al concluded that smoking was not correlated with plaque burden in 897 consecutive patients undergoing IVUS investigation (19). Our findings concur with the latter study. The lack of a substantial association between smoking and plaque burden as well as plaque volume in our study, may in part be due to the fact that our study population consisted of patients who had an indication for coronary angiography. Therefore, in never smokers, other factors (not reflected by the 'established' risk factors described in the baseline characteristics) may potentially have contributed to their burden of atherosclerosis. And as reflected by our analyses, the disease burden caused by these factors may have been similar to the disease burden caused by smoking, thus potentially obscuring the effect of smoking.

Our results do not provide support for the hypothesis that smoking is associated with coronary plaque vulnerability. Lack of such an association may in part be explained by the possibility that plaque erosion, and not as much vulnerable plaque rupture, is the intermediate between smoking and cardiac adverse events, as suggested by earlier, histopathological studies. Such studies have shown that luminal thrombosis may result from two different pathologies, namely plaque rupture and plaque erosion (8, 22-24).

Plaque rupture is highly associated with a lipid-rich atheroma with only a thin fibrous layer of intimal tissue covering the necrotic core (a thin-cap fibroatheroma, TCFA) and causes thrombotic coronary occlusion. This mechanism is the most common cause of myocardial infarction and death from cardiac causes (22). Plaque erosion is characterized by an acute thrombus in direct contact with the intimal plaque rich in smooth muscle cells with surrounding proteoglycan matrix and minimal inflammation. The lesions tend to be eccentric, infrequently calcified and cause less severe narrowing at sites of thrombosis (8, 23). Most erosion lesions have an absent or poorly defined necrotic core, which, when present, is not in close proximity to the luminal thrombus. The risk factors for erosion are poorly understood and are different from those of rupture. The literature has shown that plaque erosion is associated with smoking and frequently causes coronary thrombosis (8, 23). This may possibly explain the general absence of an association of smoking with coronary plaque composition as assessed by VH-IVUS in the literature, and the inconsistent findings with regard to smoking and degree of coronary atherosclerosis as assessed by grayscale IVUS. In the present study, we found that current smokers tend to have a slightly lower percentage fibrous plaque volume and that they have a somewhat higher percentage fibro-fatty plaque volume; however, this trend did not persist with regard to percentage necrotic core or presence of TCFA. These findings do not preclude plaque erosion as the underlying mechanism. Moreover, histopathological studies examing coronary arteries suggest that smoking predisposes patients to coronary thrombosis rather than promoting the progression of atherosclerosis (8-11). These investigations are supported by clinical patient studies showing that smokers seem to have a more favourable response to fibrinolytic therapy compared to nonsmokers, which may be attributed to their hypercoagulable state (25-27). In the present study, we did not focus on the influence of smoking on blood coagulation.

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

Some aspects of this study warrant consideration. VH-IVUS imaging took place of a prespecified single target segment of a single non-culprit coronary artery, under the assumption that such a non-stenotic segment would adequately reflect coronary wall pathophysiology of the larger coronary tree. Although this assumption may be debated, previous studies evaluating IVUS have demonstrated that the coronary wall of comparable non-culprit, non-stenotic segments of a single vessel does reflect larger coronary disease

burden and is associated with subsequent events (29, 30). Furthermore, it is important to note that IVUS is formally not capable of detecting the TCFA according to histopathological definitions (31, 32), because the spatial resolution of IVUS is insufficient for thin cap detection (31, 32). Nonetheless, a concept of VH-IVUS derived TCFA has been postulated for plaques with a plaque burden ≥ 40% and a confluent necrotic core ≥ 10% in direct contact with the lumen in at least three VH-IVUS frames (18, 31). Notably, we have recently demonstrated that such VH-IVUS derived TCFA lesions are strongly and independently predictive of the occurrence of major adverse cardiac events within the current study population (33). Another important limitation of this cross-sectional study is that smoking status was determined by self-report. To minimize the risk of misclassification, we excluded former smokers from our study. Finally, a matching procedure was necessary because of differences in baseline characteristics between smokers and never smokers. Since part of the smokers (n=29) could not be matched to a never smoker, this study design entailed some loss of statistical power.

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

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PART IV |

The role of biomarkers in prediction of clinical outcome in heart failure and after heart transplantation

6

Blood biomarkers in heart failure patients

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Blood biomarkers in heart failure patients

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Overview of the role of blood biomarkers in heart failure with normal ejection fraction

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ABSTRACT

Aims: Heart failure with normal ejection fraction (HFNEF) is a major and growing public health problem, currently representing half of the heart failure burden. Although many studies have investigated the diagnostic and prognostic value of new biomarkers in heart failure, limited data are available on biomarkers other than natriuretic peptides in HFNEF. We performed a systematic review of epidemiologic studies on the associations of biomarkers with the occurrence of HFNEF and with the prognosis of HFNEF patients.

Methods and results: Biomarkers examined most extensively in HFNEF include biomarkers of myocyte stress, inflammation and extra-cellular matrix remodeling. Some biomarkers have been shown to be increased to a different extent in HFNEF compared to heart failure with reduced ejection fraction (HFREF). Several biomarkers, including biomarkers of myocyte stress, inflammation, extracellular matrix remodeling, growth differentiation factor 15 (GDF-15), cystatin C, resistin and galectin-3 were associated with development of HFNEF and with clinical outcomes of HFNEF patients in terms of morbidity and mortality.

Conclusion: Several biomarkers, including biomarkers of myocyte stress, inflammation, extracellular matrix remodeling, growth differentiation factor 15 (GDF-15), cystatin C, resistin and galectin-3 appeared to be promising diagnostic and prognostic tools in patients with HFNEF. Investigation of the incremental diagnostic and prognostic value of these biomarkers, or a combination thereof, over established clinical covariates and imaging techniques in large, prospective studies is warranted.

INTRODUCTION

Heart failure (HF) is a major and growing public health problem that is associated with substantial morbidity and mortality.[1, 2] Classically, HF has been considered to be associated with impaired cardiac contractility and cardiac dilatation. In the past decade, however, it has become evident that a considerable portion of patients presenting with clinical HF have a normal left ventricular ejection fraction (LVEF). Some studies report a prevalence as high as 50%.[3] This entity is often termed HF with normal ejection fraction (HFNEF), sometimes also referred to as HF with preserved ejection fraction.[1, 2] Despite improvements in understanding the underlying disease mechanisms, the exact mechanism and the classification of HFNEF are still debated.[3] In the single syndrome hypothesis, HFNEF and HF with reduced LVEF (HFREF) are viewed as two ends of one HF spectrum, the major difference being the degree of left ventricular dilatation and shape change or left ventricular remodelling.[3] Although HFNEF is typically characterized by the presence of diastolic dysfunction, HFREF is found to be associated with reduced myocardial tissue doppler velocities as well, which supports the single HF syndrome hypothesis. On the other hand, the theory has been proposed that clinical HF presents and evolves not as a single syndrome but as two syndromes, one with depressed LVEF and the other with normal LVEF and specific mechanisms responsible for diastolic LV dysfunction, this theory being supported by structural, functional, and molecular biological arguments.[3] Regardless of which hypothesis will eventually turn out to be appropriate, the prognosis after hospitalization for HFNEF appears to be as ominous as that of HFREF with a mortality rate of approximately 65% at 5 years.[4]

Although echocardiography is the most useful noninvasive diagnostic method for evaluating systolic and diastolic dysfunction, current state-of-the-art echocardiography has limited value for prognostication in HF.[1, 2] HF results from a complex interplay between genetic, neurohormonal, inflammatory and biochemical changes acting on cardiac myocytes and the cardiac interstitium. Thus, the sequence of events that lead to overt changes in the ventricle begins at cellular level, and assessing these phenomena could be of greater value to improve prognostication. Biomarkers, in this context meaning proteins measured in blood, may play an important part in this respect. Biomarkers may provide important information on the pathogenesis of HF, but may also be a valuable clinical tool in the identification of patients at risk for HF, in the diagnosis of HF, in risk stratification and in monitoring therapy. Furthermore, reliable non-invasive measures of pre-symptomatic worsening of (diastolic) ventricular function, including biomarkers, could aid in prevention of ensuing decompensation with its adverse sequels.

Although many studies have investigated the diagnostic and prognostic value of new biomarkers in HF, the majority of these studies included HFREF patients only.[5] Limited data are available on biomarkers in HFNEF. This review provides a thorough, yet concise, overview of clinical and population based studies on the associations of biomarkers with

occurrence of HFNEF and with prognosis of HFNEF patients, and of the pathophysiology underlying these associations. We summarize research on established biomarkers, such as natriuretic peptides, but we mainly focus on biomarkers of myocyte stress, inflammation and extra-cellular matrix remodeling since the body of evidence on these markers is less elaborate.

METHODS

Using Medline (Pubmed U.S. National Library of Medicine), we performed a literature search from inception to February 2012 using the following search terms: “Heart Failure” (MeSH term) and “normal ejection fraction” or “HFNEF” or “preserved ejection fraction” or “HFPEF” or “diastolic heart failure” or “DHF” or “diastolic dysfunction” in combination with “Biological Markers” (MeSH term) or “cytokine” or “CRP” or “TNF” or “MMP” or “TIMP” or “collagen”. We limited our search to studies on human adults. Articles were included if they fulfilled the following criteria: a study population that includes patients with HFNEF or that has registered incident HFNEF; measurement of biomarkers in blood samples (other than natriuretic peptides); and reference to outcome in terms of morbidity and mortality. In addition, references of included studies were checked to ensure that no potentially eligible studies were missed.

HFNEF was defined as a reported clinical diagnosis of HF as well as LVEF higher than a cutoff value of choice in the specific study, which could range from 40% to 55%. Studies that have reported on associations with diastolic dysfunction in general, but not on associations with HFNEF, were excluded.

RESULTS

The systematic literature search yielded 198 potential eligible studies. After exclusion of the studies that did not fulfill our criteria, 26 original studies were included in this review. The study populations and baseline characteristics of these studies are shown in Table 1. Additionally, three literature reviews were found on the utility of natriuretic peptides in HFNEF.[6-8]

BIOMARKERS OF MYOCYTE STRESS

Natriuretic peptides

Among biomarkers of myocyte stress, brain natriuretic peptides (BNP) have been investigated most extensively.[5] Nevertheless, our understanding on the biochemistry of natriuretic peptides currently may not be fully complete, as exemplified by the occurrence

of the "natriuretic peptide paradox". Pro-brain natriuretic peptide (proBNP) is synthesized by the heart in reaction to cardiac wall distension and stretching, and neurohormonal activation.[9] During secretion from the cardiomyocytes, the biologically inactive amino-terminal fragment (NT-proBNP) is split from proBNP. An increased active BNP concentration in the plasma leads to natriuresis, vasodilatation, inhibition of the renin-angiotensin system, adrenergic activity and improved myocardial relaxation. Herewith, BNP is expected to have an important regulatory role in response to acute increases in ventricular volume and overload. In HF patients, although levels of serum immunoreactive natriuretic peptides are already elevated, administration of BNP has additional beneficial effects.[10] This "natriuretic peptide paradox" may be explained by the fact that in HF patients, the fraction of active BNP in the blood is relatively small. This signifies that HF is characterized by altered natriuretic peptide processing with secretion of less biologically active forms, while proBNP is the major immunoreactive form that is measured by laboratory assays.[10]

The associations of the natriuretic peptides with HFNEF have already been evaluated extensively in recently published reviews.[6-8] As such, we will not evaluate these associations in depth in the current paper. In brief, the majority of data show that BNP and NT-proBNP levels are increased in both HFREF and HFNEF compared to control subjects. Higher plasma NT-proBNP levels are shown to be associated with greater severity of diastolic dysfunction in patients with HFNEF.[11] However, co-morbidities are major drivers of higher NT-proBNP levels in HFNEF as well.[12] Several studies have also shown that plasma levels of natriuretic peptides are strong predictors of mortality and hospitalizations in both patients with HFREF and patients with HFNEF.[13] For a given BNP level, the prognosis in patients with HFNEF is as poor as in those with HFREF.[14]

In current clinical guidelines, the recommended natriuretic peptide cutoff values for the diagnosis of HF do not differ between HFREF and HFNEF.[1, 2] However, there are some interesting differences in the epidemiology of natriuretic peptides between the HFREF and the HFNEF populations. Firstly, the increase of NT-proBNP levels is less pronounced in HFNEF.[6-8] NT-proBNP levels have been shown to be lower in HFNEF than HFREF patients of a similar NYHA class.[15] Furthermore, patients in the Irbesartan in heart Failure with Preserved Ejection Fraction Trial (I-PRESERVE) had low overall NT-proBNP levels.[16] These relatively lower natriuretic peptide levels suggest a lower diastolic wall stress in HFNEF when compared to HFREF. In case NT-proBNP is required to be elevated for a definite diagnosis of HF, only the higher-risk HFNEF patients will be identified, resulting in a reduced prevalence of HFNEF.[17] Secondly, the strategy of using elevated natriuretic peptides concentrations as a patient selection criterion for HFNEF trials could be questioned because the I-PRESERVE trial showed that the use of irbesartan was associated with improved outcomes in patients with NT-proBNP below, but not above, the median concentration.[16]

Table 1. Baseline characteristics

	Year	Population (n)	EF cut-off for HFNEF	Sample size, n				Age, years ± SD			
				HFNEF	*HFREF*	*LVH, no HF*	*No HF or LVH*	*HFNEF*	*HFREF*	*LVH, no HF*	*No HF or LVH*
Yu et al. (15)	2001	hospitalized HF (77), healthy controls (17)	50%	31	46		17	66.5 ± 8.4	65.7 ± 12.2		
Amosova et al. (23)	2004	hospitalized HFNEF (26), healthy controls (10)	NR								
Wisniacki et al. (19)	2005	outpatient HF aged 70-90 (52), healthy (26)	50%	25	27		26	80.4 ± 4.5	79.8 ± 5.2		76,1 ± 3.5
Ahmed et al. (35)	2006	outpatient LVH patients (49), healthy controls (53)	50%	26		23	53				59 ± 7
Martos et al. (36)	2007	outpatient HT patients (86)	45%	32			54	72 ± 11			67 ± 9
Varol et al. (49)	2007	outpatient HCM patients with HF (32), healthy controls (30)	NR	32			30	51.3 ± 18.4			49.6 ±16.1
Frantz et al. (37)	2008	hospitalized HF (249), healthy controls (74)	45%	102	147		74				
Michowitz et al. (17)	2008	outpatient HF (294), healthy controls (7701)	45%	77	217		7701	71 ± 11.2	72.4 ± 10.8		
Dunlay et al. (29)	2008	community-based HF patients (486)	50%	486				76.7 ± 13.0			
Moran et al. (44)	2008	community-based aged ≥65 (4453)	50%				4453				
Niethammer et al. (24)	2008	hospitalized HFNEF (17), hospitalized HFPEF (17), healthy controls (20)	50%	17	17		20	72 ± 9	70 ± 8		56 ± 5
Barasch et al. (40)	2009	community-based aged ≥65 (880)	55%	179	131		570	76 ± 5	77 ± 6		77 ± 6
Butler et al. (46)	2009	community-based aged 70-79 (2902)	40%				2902				73.6 ± 2.9
Naito et al. (38)	2009	hospitalized HF (110), hospitalized non-HF patients (10)	45%	42	68		10	74 ± 13	71 ± 16		70 ± 13
Okuyan et al. (18)	2010	hospitalized HFNEF (68), healthy controls (40)	50%	68			40	65.5 ± 9.6			65.2 ± 9.7
Stahrenberg et al. (42)	2010	community-based HF patients (228), healthy elderly controls (188)	50%	142	86		188	73 [66-78]	71 [66-75]		56 [52-63]
Gonzales et al. (39)	2010	outpatient hypertensive patients with HFNEF (156), healthy controls (20)	50%	156			20	75 ± 9			
Kalogeropoulos et al. (28)	2010	community-based aged 70-79 (2610)	45%				2610				73.6 ± 2.9
De Boer et al. (13)	2011	Hospitalized HF (592)	40%	114	368			74 ± 10	69 ± 12		
Matsubara et al. (16)	2011	hospitalized HF (181), hospitalized non-HF patients (171)	50%	82	70		171	71.2 ± 10.2	65.5 ± 13.6		66.5 ± 11.2
Carrasco-Sanchez et al. (45)	2011	hospitalized HFNEF (218)	45%	218				75.6 ± 8.7			
Wu et al. (25)	2011	hospitalized HFNEF (110), hospitalized non-HF patients (55)	NR	110			55	72.22 ± 9.86			72.16 ± 9.62
Zile et al. (26)	2011	outpatient LVH patients (205), healthy controls (241)	50%	61		144	241	66 ± 8		60 ± 12	58 ± 16
Collier et al. (27)	2011	outpatient HT patients (275)	50%	181			94	73 ± 12			66 ± 10
Krum et al. (41)	2011	outpatient HFNEF aged ≥60 (313)	45%	313				72 ± 7			
Santhanakrishnan et al. (43)	2012	In- and outpatient compensated HF patients (101), healthy controls (50)	50%	50	51		50	69 ± 12	59 ± 11		63 ± 8

Data are presented as percentage, mean ± standard deviation or as median [interquartile range].
DM = diabetes mellitus; EF = ejection fraction; HF = heart failure; HFNEF = heart failure with normal ejection fraction; HFREF = heart failure with reduced ejection fraction; HT = hypertension; LVF = left ventricular hypertrophy; NR = not reported; NYHA = New York Heart Association.

Male gender, %				**DM, %**				**HT, %**				**Ischaemic heart disease, %**				**NYHA III/IV, %**	
HFNEF	*HFREF*	*LVH, no HF*	*No HF or LVH*	*HFNEF*	*HFREF*	*LVH, no HF*	*No HF or LVH*	*HFNEF*	*HFREF*	*LVH, no HF*	*No HF or LVH*	*HFNEF*	*HFREF*	*LVH, no HF*	*No HF or LVH*	*HFNEF*	*HFREF*
81	72											87.9	84.1			16.7	22.9
48	59.3		53.8													52	44
			38														
53			76	12			7	100			100						
66			63	13			0					13			0	34	
61	81.1			40.2	38.2			70.1	57.1			50.6	83.4				
48.6				30.5				80.4				53.7				73.0	
47	82		45	18	24		0	100	88		13						
45	63		52	27	30		11	53	48		29	66	83		14		
			48.1				14.7				43.5				16.5		
52	0.64		60	33	32		30	48	31		50	17	50		70		
43			40	51.47			30	66.17			60						
36	83		34	30	37		0	93	91		1	35	52		0		
46								100									
			48.3				14.8				53.1				19.5		
50	66			29	28			51	40			30	44			47	59
72	67		57	45.1	28.6		37.4	72.0	54.3		67.8	53.7	45.7		55.0	37	63
39.9				52.8				83.5				18.8				39.4	
52			26	29			36	69			78						
41		45	30	31		16	9										
54			55	19			14	100			100	38			30		
34				34				96				21				83	
58	86		46	40	49		3	88	69		36	32	59		0	16	37

The above considerations suggest that natriuretic peptides may be less useful as a diagnostic and prognostic tool in HFNEF than in HFREF. Although NT-proBNP has a better negative predictive value than invasive measurement of left ventricular end-diastolic pressure, tissue doppler imaging indices and conventional echocardiography measurements in HFNEF patients, the overall diagnostic performance of NT-proBNP according to the receiver operating characteristic was only similar to that of tissue doppler imaging indices.[11] This calls for further exploration of other biomarkers which may provide incremental value specifically in HFNEF. The value of such other biomarkers will be discussed in this review.

Other biomarkers of myocyte stress

Another interesting biomarker of myocyte stress is adrenomedullin. Adrenomedullin is a hormone that lowers systemic vascular resistance and has natriuretic and diuretic effects. It is produced in several organs such as the heart, lungs and kidneys. We found one study that examined adrenomedullin in HFNEF (Table 4). Yu et al. showed that plasma adrenomedullin concentrations were higher in HFNEF patients than in healthy controls.[18] There was no significant difference in adrenomedullin concentration between HFNEF and HFREF patients. Furthermore, the authors concluded that adrenomedullin concentrations in patients with HF are especially raised in the presence of a restrictive filling pattern. The incremental value of adrenomedullin over natriuretic peptides has not yet been investigated.

BIOMARKERS OF INFLAMMATION

Pathophysiology

Biomarkers of inflammation are among the first to have been linked to HF. Early studies have focused on tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP). These proinflammatory cytokines probably contribute to the clinical syndrome of HF and to progression of the disease through adverse effects on the vascular endothelium, myocyte apoptosis, induction of hypertrophy (e.g. by IL-6) and left ventricular dilatation (e.g. by TNF-α).[5] CRP has been correlated with the severity and prognosis of HF, as well as with response of HF patients to treatment.[19-22]

Inflammatory biomarkers may have different meanings in HFREF and HFNEF. In HFREF, inflammatory biomarkers may be a measure of heart failure severity. Although the source of cytokine production in HFREF is still unknown, it may possibly reside in the failing myocardium itself because of hemodynamic overload.[23] Alternatively, it may result from extramyocardial production in the bowel, because of altered tissue perfusion and tissue hypoxia, possibly modulated by bacterial endotoxin release from the gut.[23] In contrast, in the case of HFNEF, inflammatory biomarkers, particularly those associated with metabolic

syndrome, may be a measure of risk for developing HFNEF. Obesity and diabetes mellitus are believed to play a major role in the remodeling of the ventricles and in the development of HFNEF.[24] Both obesity and diabetes mellitus are associated with increased inflammatory biomarker levels. As such, inflammatory biomarker levels may be a measure of factors driving left ventricular remodeling in HFNEF.

Moreover, a high body mass index appears to be beneficial in HFREF patients. Explanations for this obesity paradox in HFREF include possible protective effects conferred by excess body weight on HF mortality - because advanced HF is a catabolic state, obese patients with HF may have more metabolic reserve - and protective effects of cytokine and neuroendocrine profiles of obese patients.[25] However, this obesity paradox is missing in HFNEF,[24, 26] which further underscores the important role of obesity in the development of this condition.

Biomarker levels in HFNEF

Although many studies have examined the associations of inflammatory biomarkers with HF in general, HFNEF has received less attention. Most of the studies that have included HFNEF patients have employed cross-sectional or case-control designs, and are summarized below (Table 2 and Supplemental Table 1).[19-22, 27-31] Several studies have demonstrated elevated TNF-α and IL-6 levels in HFNEF patients when compared to a non-HF reference group.[19, 22, 28, 29, 31] Also, the concentrations of the TNF-α receptors (sTNFR-1 and sTNFR-2) were found to be higher in HFNEF patients.[22, 28] Serum levels of CRP and Pentraxin-3 (PTX-3), a relatively newly-identified acute phase protein of the pentraxin superfamily which also includes CRP, were both found to be significantly higher in HFNEF patients when compared to with the non-HF reference group.[19-22] Finally, Collier et al. identified IL-8 and monocyte chemoattractant protein-1 (MCP-1) as novel inflammatory biomarkers of HFNEF.[31] These results once again emphasize that inflammatory biomarkers may play an important role in the development and progression of HFNEF.

Biomarker levels in HFNEF compared with HFREF

A few of the above mentioned studies also compared inflammatory marker concentrations in HFNEF with HFREF. Regarding this comparison, the results were less consistent between studies. Wisniacki et al. reported no significant difference in CRP, sTNFR2 and IL-6 levels between 25 HFNEF patients and 27 HFREF patients.[22] Michowitz et al. did not find a significant difference in CRP elevation between HFNEF and HFREF patients either.[20] Niethammer et al. showed that TNF-α and IL-10 were significantly elevated in HFREF but not in HFNEF.[28] Furthermore, sTNFR1 levels were less highly elevated in HFNEF than in HFREF, while sTNFR2 levels were similarly elevated in both groups. Matsubara et al. demonstrated less elevation of CRP, PTX3 and IL-6 levels in HFNEF compared to HFREF,

while TNF-α concentrations were similarly elevated.[19] These findings suggest that several inflammatory biomarkers are more pronounced in HFREF, which supports the hypothesis that the origin and the meaning of these markers may differ between HFREF and HFNEF.

Association with incidence of HFNEF

Kalogeropoulos et al. examined 2610 persons aged 70-79 years from the Health ABC study and found that TNF-α (unadjusted HR per doubling 1.48; 95% CI 1.19-1.86) and IL-6 (unadjusted HR per doubling 1.81; 95% CI 1.23-2.68) were associated with incident HFNEF during follow-up.[32] Such associations of TNF-α and IL-6 were less strong for HFREF. CRP was not found to be a significant predictor. These findings support the hypothesis that inflammatory biomarkers are particularly associated with development of HFNEF.

Association with HFNEF prognosis

Few follow-up studies have been performed on the role of inflammatory biomarkers in HFNEF. Michowitz et al. found that CRP was independently associated with hospitalization in patients with HFREF, but not in patients with HFNEF.[20] These results support the hypothesis that inflammatory biomarkers are a measure of heart failure severity, particularly in HFREF. Mortality was not predicted by CRP levels in either patient category.
Within the Olmsted County study, Dunlay et al. performed a prospective study in which they examined 486 patients with active HF of whom 54% had HFNEF.[33] They found that mortality increased with increasing TNFα level (unadjusted HR of highest vs lowest quartile 2.10; 95% CI 1.30-3.38). No interaction was present between TNFα and ejection fraction, thus implying the effect was similar in HFNEF and HFREF.

BIOMARKERS OF EXTRACELLULAR-MATRIX REMODELING

Pathophysiology

The extracellular matrix provides a skeleton for myocytes and influences their size and shape.[5] Changes in the extracellular matrix may be causally related to remodeling of the ventricles resulting in progression of HF.[5] Collagen turnover in the extracellular matrix is mainly regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). The MMPs are a family of endopeptidases that digest interstitial constituents. The various MMPs have different substrates. TIMPs are proteins that bind to and inhibit the effects of MMPs. Furthermore, carboxy-terminal propeptide of procollagen type I (PICP), amino-terminal propeptide of procollagen type I (PINP) and type III (PIIINP) are markers of collagen biosysthesis, while carboxy-terminal telopeptide of collagen type I (CITP) is a marker of collagen degradation.

Histological research has demonstrated that the nature of fibrosis differs between HFNEF and HFREF.[34] Fibrosis in HFNEF is mainly interstitial, while fibrosis in HFREF is both interstitial and replacement fibrosis.[34] Therefore, it may be expected that different pathophysiological pathways are activated and that different profiles of collagen biomarkers are expressed in HFNEF versus HFREF. Previous studies have shown that interstitial fibrosis is associated with increased expression of TIMPs,[35] while replacement fibrosis is associated with increased expression of MMPs.[36] As such, it may be expected that TIMPs are particularly upregulated in HFNEF, while several MMPs are particularly upregulated in HFREF. Furthermore, in contrast to HFREF, myocardial stiffness may even be more important than extracellular fibrosis as a mechanism for diastolic stiffness in HFNEF.[37] HFNEF patients with only mild elevations of collagen volume fraction may have highly elevated left ventricular end-diastolic pressures.[38] Unfortunately, no biomarkers have been identified yet in order to measure this myocardial stiffness.

Biomarker levels in HFNEF

Most studies have applied a cross-sectional or case-control design (Table 3 and Supplemental table 2).[30, 31, 39-43] Although some of the results were not consistent between studies, most of the studies showed that MMP-1, MMP-2, MMP-9, TIMP-1, PICP, PIIINP and CITP were elevated in HFNEF patients when compared to a control group consisting of patients without HF.[30, 31, 39-43] Also MMP-3, MMP-7, TIMP-4 and Osteopontin concentrations might be elevated in HFNEF, but these biomarkers are less well investigated.[30] Interestingly, lower serum concentrations of MMP-8 and MMP-13 were observed in patients with HFNEF.[30, 39] Furthermore, Gonzalez et al. observed that the MMP-1/TIMP-1 ratio was increased in HFNEF patients with normal left-sided filling pressures.[43] These findings support the hypothesis that the balance of collagen turnover is disturbed in patients with HFNEF.

Biomarker levels in HFNEF compared with HFREF

Only two studies compared biomarker levels of HFNEF with HFREF patients. Frantz et al. reported similarly elevated TIMP-1 levels in hospitalized HFNEF and HFREF patients. Naito et al. found similarly elevated plasma concentration of MMP-2 in hospitalized HFNEF and HFREF patients. MMP-1 concentration, however, was significantly higher in HFREF patients. The observed difference in MMP-1 concentration supports the hypothesis that different profiles of extracellular-matrix biomarkers are expressed in HFREF and HFNEF.

Table 2. Biomarkers of inflammation

	Index group (n)	*Reference group (n)*	*Outcome*	CRP	PTX-3	TNF-α	sTNFR1	sTNFR2	IL-6	Cardio-trophin-1	IL-8	IL-10	MCP-1
Cross-sectional / case-control studies comparing HFNEF with a non-HF reference group													
Amosova et al. (23)	hospitalized HFNEF (26)	healthy (10)	level			↑			↑				
Wisniacki et al. (19)	outpatient HFNEF (25)	healthy (26)	level	↑				↑	↑				
Michowitz et al. (17)	outpatient HFNEF (77)	healthy (7701)	level	↑									
Niethammer et al. (24)	hospitalized HFNEF (17)	healthy (20)	level			ns	↑	↑	↑			ns	
Okuyan et al. (18)	hospitalized HFNEF (68)	healthy (40)	level	↑									
Matsubara et al. (16)	hospitalized HFNEF (82)	non-HF patients (171)	level	ns	↑	↑			↑				
Wu et al. (25)	hospitalized HFNEF (110)	non-HF patients (50)	level			↑			↑				
Zile et al. (26)	outpatient HFNEF (61)	healthy (241)	level							ns			
	outpatient HFNEF (61)	LVH without HF (144)	level							ns			
Collier et al. (27)	outpatient HFNEF (181)	HT patients without HF (94)	level			ns			↑		↑		↑

Table 2. Continued

	Index group (n)	*Reference group (n)*	*Outcome*	CRP	PTX-3	TNF-α	sTNFR1	sTNFR2	IL-6	Cardio-trophin-1	IL-8	IL-10	MCP-1
Cross-sectional / case-control studies comparing HFNEF with HFREF													
Wisniacki et al. (19)	outpatient HFNEF (25)	outpatient HFREF (27)	level	ns				ns	ns				
Michowitz et al. (17)	outpatient HFNEF (77)	outpatient HFREF (217)	level	ns									
Niethammer et al. (24)	hospitalized HFNEF (17)	hospitalized HFREF (17)	level			↓	↓	ns	ns			↓	
Matsubara et al. (16)	hospitalized HFNEF (82)	hospitalized HFREF (70)	level	↓	↓	ns			↓				
Follow-up studies													
Michowitz et al. (17)	outpatient HFNEF (77)		Hospitalization (2.8y follow-up)	ns									
			Mortality (2.8y follow-up)	ns									
Dunlay et al. (29)	community-based HF patients* (486)		Mortality (1y follow-up)			HR 2.10 (1.30-3.38)†							
Kalogeropoulos et al.(28)	community-based aged 70-79 (2610)		HFNEF (9.4y follow-up)	ns		HR 1.81 (1.23-2.68)‡			HR 1.48 (1.19-1.86)‡				

↑ Indicates a significantly higher biomarker level in the index group compared to the reference group. ↓ Indicates a significantly lower biomarker level in the index group compared to the reference group. HF = heart failure; HFNEF = heart failure with normal ejection fraction; HFREF = heart failure with reduced ejection fraction; CRP = C-reactive protein; HT = hypertension; IL = interleukin; LVF = left ventricular hypertrophy; MCP-1 = monocyte chemoattractant protein 1; ns = not significant; PTX-3 = pentraxin 3; TNF-α = tumor necrosis factor α; sTNFR = soluble tumor necrosis factor receptor.
* Of whom 54.8% had HFNEF.
† Unadjusted hazard ratio of highest quartile compared to lowest quartile.
‡ Unadjusted hazard ratio per doubling.

Association with incidence of HFNEF

Barasch et al. performed a nested case-control study within the Cardiovascular Health study, which has longitudinal follow-up.[44] Biomarkers were assessed at 5-year or 9-year follow-up in a total of 880 subjects (131 with systolic HF, 179 with HFNEF, 280 controls with cardiovascular risk factors and 279 healthy controls). In the total study population, elevated CITP (OR per tertile 3.1; 95% CI 2.4 to 4.0) and PIIINP (OR per tertile 2.2; 95% CI 1.7 to 2.8) were associated with incident HFNEF during follow-up.

Association with HFNEF prognosis

Follow-up studies on the prognostic utility of biomarkers of extracellular matrix remodeling are also small in number. Within the Irbesartan in Heart Failure With Preserved Systolic Function (I-PRESERVE) trial, Krum et al. investigated the prognostic value of PINP, PICP and Osteopontin during follow-up of 4.1 years.[45] In univariable analysis, increased levels of these biomarkers were all associated with the composite endpoint of mortality and hospitalization, all-cause mortality and the composite of HF related death or hospitalization. However, none of these biomarkers remained significant as an independent predictor when introduced into a multivariable model adjusting for 19 clinical parameters. These results are in line with above-mentioned findings suggesting that myocardial stiffness is a more important factor of diastolic dysfunction than fibrosis in patients with HFNEF.

OTHER BIOMARKERS

Homocysteine

Homocysteine is traditionally believed to have pro-oxidative, pro-inflammatory and vasoconstrictive properties, and to cause endothelial vascular dysfunction. Experimental studies have demonstrated that elevated homocysteine levels may also adversely affect the myocardium, leading to pathological hypertrophy of ventricles with disproportionate increase in collagen.[21] Okuyan et al. measured homocysteine concentrations in 68 hospitalized HFNEF patients and 40 healthy controls (Table 4 and Supplemental table 3).[21] Homocysteine concentrations were significantly higher in HFNEF patients. The authors state that pathologic mechanisms and effects of homocysteine on the natural history of HF still need to be clarified.

Growth differentiation factor 15

Growth differentiation factor 15 (GDF-15) is suggested to be a downstream marker indicative of different pathways of myocardial stress and inflammation. In animal models, GDF-15 was found to attenuate reduction in fractional shortening and to protect the heart

from hypertrophy and ischemia-reperfusion injury.[46] Stahrenberg et al. measured GDF-15 in 142 HFNEF patients, 86 HFREF patients and 188 healthy elderly controls.[46] They found that GDF-15 was higher in HFNEF compared to controls. Serum GDF-15 concentrations were equal in HFNEF and HFREF patients. The authors concluded that diagnostic precision of GDF-15 was at least as good as that of NT-proBNP, and that a combination significantly improved diagnostic accuracy. Similar results were reported by Santhanakrishnan et al.[47]

Cystatin C

Renal function is also believed to play a role in the evolvement of HF.[48, 49] Cystatin C is a marker of renal function. Moran et al examined 4453 subjects aged 65 years or older without HF at baseline from the Cardiovascular Health Study.[48] They compared the association of cystatin C with risk of incident HFNEF and HFREF.

During 8 years of follow-up, 167 cases of incident HFNEF and 206 cases of incident HFREF occurred. Increased risk of HFNEF was apparent only in the highest cystatin C quartile (HR 2.25; 95% CI 1.33-3.80), while a linear trend was present for HFREF. This study demonstrates that kidney dysfunction is a risk factor for occurrence and progression of HF. Carrasco-Sanchez et al. investigated the prognostic value of cystatin C in HFNEF.[49] They included 218 hospitalized HFNEF patients and collected 1-year follow-up. Cystatin C was a strong predictor of the composite of mortality or hospitalization (HR of highest compared to lowest quartile 4.85; 95% CI 2.76-8.51) and mortality alone (HR 11.35; 95% CI 4.01-32.14). Cystatin C also remained a strong independent predictor with multivariable analysis.

Resistin

Another potentially interesting biomarker is resistin. Resistin has been found to be produced and released from adipose tissue. Although the exact function of resistin is not known, it has been associated with insulin resistance and inflammatory response.[50] Resistin concentrations have previously been correlated with risk of coronary artery disease, renal dysfunction and adverse outcomes among stroke patients.[50] Butler et al. measured resistin in 2902 subjects aged 70-79 years without prevalent HF from the health ABC study, where after these patients were followed-up for incident HF.[50] Resistin was found to be associated with both incident HFNEF (HR per 10ng/mL 1.42; 95% CI 1.27-1.58) and incident HFREF (HR per 10ng/mL 1.35; 95% CI 1.20-1.53). The prognostic value of resistin in HFNEF patients has not been investigated yet. Another study showed that leptin, which is also a biomarker related to adiposity and metabolic syndrome, is associated with incidence of HF.[51] A third biomarker in this category, adiponectin, was not found to be associated with HF.[52] Overall, these findings are in line with the hypothesis that obesity and the metabolic syndrome drive development of HF.

Table 3. Biomarkers of extracellular matrix remodeling

	Index group (n)	*Reference group (n)*	*Outcome*	**MMP-1**	**MMP-2**	**MMP-3**	**MMP-7**	**MMP-8**
Cross-sectional / case-control studies comparing HFNEF with a non-HF reference group								
Ahmed et al. (35)	outpatient HFNEF (26)	healthy without HT (39)	level		ns			
	outpatient HFNEF (26)	healthy with HT (14)	level		ns			
	outpatient HFNEF (26)	LVH without HF (23)	level		ns			
Martos et al. (36)	outpatient HFNEF (32)	HT patients without HF (54)	level	ns	↑			
Frantz et al. (37)	hospitalized HFNEF (102)	healthy (74)	level					
Naito et al. (38)	hospitalized HFNEF (42)	non-HF patients (10)	level	↑	↑			
Gonzales et al. (39)	outpatient HFNEF (156)	healthy (20)	level	↑				
Zile et al. (26)	outpatient HFNEF (61)	healthy (241)	level	ns	↑	ns	↑	ns
	outpatient HFNEF (61)	LVH without HF (144)	level	ns	↑	↑	ns	↓
Collier et al. (27)	outpatient HFNEF (181)	HT patients without HF (94)	level		↑			
Cross-sectional / case-control studies comparing HFNEF with HFREF								
Frantz et al. (37)	hospitalized HFNEF (102)	hospitalized HFREF (147)	level					
Naito et al. (38)	hospitalized HFNEF (42)	hospitalized HFREF (68)	level	↓	ns			
Follow-up studies								
Barasch et al. (40)	community-based aged ≥65 (880)		HFNEF (4y follow-up)					
Krum et al. (41)	outpatient HFNEF aged ≥60 (313)		mortality or hospitalization (4.1y follow-up)					
			all-cause mortality (4.1y follow-up)					
			HF death or hospitalization (4.1y follow-up)					

;a significantly lower biomarker level in the index group compared to the reference group. CITP = carboxy-terminal telopeptide of collagen type I; HF = heart failure; HFNEF = heart failure with normal ejection fraction; HFREF = heart failure with reduced ejection fraction; HT = hypertension; LVF = left ventricular hypertrophy; MMP = matrix metalloproteinasis; ns = not significant; PICP = carboxy-terminal propeptide of procollagen type I; PIIINP = amino-terminal propeptide of procollagen type III; PINP = amino-terminal propeptide of procollagen type I; TIMP = tissue inhibitor of metalloproteinasis.

* Unadjusted odds ratio per tertile.

† Unadjusted hazard ratio per 10 µg/L.

‡ Unadjusted hazard ratio per 10 nmol/L.

MMP-9	MMP-13	TIMP-1	TIMP-2	TIMP-3	TIMP-4	PINP	PICP	PIIINP	CITP	Osteopontin
↑	↓	↑	ns							
↑	↓	↑	ns							
ns	ns	↑	ns							
↑		ns				ns	↑	↑	↑	
ns		↑								
		↑					↑			
↑		↑	↑	ns	↑	ns		↑	↑	↑
ns		ns	ns	ns	↑	ns		↑	ns	ns
↑		ns				ns	ns	↑	↑	
		ns								
							ns	OR 2.2 (1.7-2.8)*	OR 3.1 (2.4-4.0)*	
						HR 1.09 (1.05-1.13)†		HR 2.47 (0.97-6.33)†		HR 1.08 (1.03-1.15)‡
						HR 1.06 (1.03-1.09)†		HR 2.85 (1.52-5.36)†		HR 1.06 (1.02-1.11)‡
						HR 1.09 (1.05-1.13)†		HR 5.91 (2.94-11.88)†		HR 1.06 (0.99-1.14)‡

Table 4. Other biomarkers

	Index group (n)	*Reference group (n)*	*Outcome*
Cross-sectional / case-control studies comparing HFNEF with a non-HF reference group			
Yu et al. (15)	hospitalized HFNEF (31)	healthy (17)	level
Wisniacki et al. (19)	oupatient HFNEF (25)	healthy (26)	level
Varol et al. (49)	outpatient HFNEF (32)	healthy (30)	level
Okuyan et al. (18)	hospitalized HFNEF (68)	healthy (40)	level
Stahrenberg et al. (42)	community-based HFNEF (142)	healthy (188)	level
Zile et al. (26)	outpatient HFNEF (61)	healthy (241)	level
	outpatient HFNEF (61)	LVH without HF (144)	level
Santhanakrishnan et al. (43)	in- and outpatient HFNEF (50)	healthy (50)	level
Cross-sectional / case-control studies comparing HFNEF with HFREF			
Yu et al. (15)	hospitalized HFNEF (31)	hospitalized HFREF (46)	level
Wisniacki et al. (19)	outpatient HFNEF (25)	outpatient HFREF (27)	level
Stahrenberg et al. (42)	community-based HFNEF (142)	community-based HFREF (86)	level
De Boer et al. (13)	hospitalized HFNEF (114)	hospitalized HFREF (368)	level
Santhanakrishnan et al. (43)	in- and outpatient HFREF (51)	in- and outpatient HFREF (51)	level
Follow-up studies			
Moran et al. (44)	community-based aged ≥65 (4453)		HFNEF (8y follow-up)
Butler et al. (46)	community-based aged 70-79 (2902)		HFNEF (9.4y follow-up)
De Boer et al. (13)	hospitalized HF (592)		mortality or hospitalization (1.5y follow-up)
Carrasco-Sanchez et al. (45)	hospitalized HFNEF (218)		mortality or hospitalization (1y follow-up)
			all-cause mortality (1y follow-up)

↑ Indicates a significantly higher biomarker level in the index group compared to the reference group. ↓ Indicates a significantly lower biomarker level in the index group compared to the reference group. CA-125 = Carbohydrate antigen 125; GDF-15 = growth differentiation factor 15; HF = heart failure; HFNEF = heart failure with normal ejection fraction; HFREF = heart failure with reduced ejection fraction; LVF = left ventricular hypertrophy; ns = not significant; sRAGE = soluble receptor for advanced glycation end-product.

* Hazard ratio of highest quartile compared to lowest quartile adjusted for age, gender and race.

† Unadjusted hazard ratio per 10ng/mL.

‡ Unadjusted hazard ratio per doubling.

§ Unadjusted hazard ratio of highest quartile compared to lowest quartile.

Adreno-medullin	Nor-adrenalin	Homo-cysteine	GDF-15	Cystatin C	Resistin	Galectin-3	sRAGE	CA-125	Troponin-T	ST2
↑										
	ns									
								ns		
		↑								
			↑							
							ns			
							ns			
			↑						↑	↑
ns										
	ns									
			ns							
						ns				
			ns						↓	ns
				HR 2.25 (1.33-3.80)*						
					HR 1.42 (1.27-1.58)†					
						HR 1.97 (1.62-2.42)‡				
				HR 4.85 (2.76-8.51)§						
				HR 11.35 (4.01-32.14)§						

Galectin-3

Galectin-3 is a protein that has a broad biological functionality. It is known to be involved in cell adhesion, cell activation, chemo-attraction, cell growth, cell differentiation, fibroblast activation and apoptosis.[15] Galectin-3 has been proposed as a novel biomarker of HF. It was found to be associated with increased risk for incident HF and mortality.[15] De Boer et al. showed that galectin-3 levels did not differ between 114 hospitalized HFNEF patients and 368 HFPEF patients.[15] In the overall HF study population, galectin-3 was found to be a significant predictor of the composite of mortality and hospitalization (HR per doubling 1.97; 95% CI 1.62-2.42). The predictive value of galectin-3 was stronger in HFNEF compared to HFREF. Furthermore, combined galectin-3 and BNP levels increased prognostic value over either biomarker alone.

Advanced glycation end-products

Advanced glycation end-products (AGEs) are formed through a reaction between proteins and sugar residues. AGEs and their soluble receptors (sRAGEs) are known to induce intracellular damage and to play a role in chronic inflammation.[30] Enhanced accumulation of AGE is thought to play a role in the pathophysiology of chronic HF. Zile et al. measured sRAGE concentration in HFNEF patients and controls. They could not detect a significant sRAGE elevation in HFNEF.[30]

Carbohydrate antigen 125

Carbohydrate antigen 125 (CA-125) is traditionally known as a tumor marker for ovarian cancer. However, non-malignant serosal effusions may display elevated serum CA125 levels as well, most likely due to increased CA-125 production by the serosal mesothelium.[53] Previously, CA125 has been shown to be elevated in HF and to be related with HF severity.[53] Varol et al. measured CA-125 concentrations in 32 HFNEF outpatients with hypertrophic cardiomyopathy and in 30 healthy controls.[53] Although the difference in CA-125 level between the groups was not significant, CA-125 levels increased with NYHA class and level of diastolic dysfunction.

Troponin-T

Troponin-T is a well-established marker of myocardial necrosis in acute coronary syndromes, but its role in HF is less well defined.[47] Santhanakrishnan et al. have evaluated several emerging biomarkers, including high sensitivity troponin-T.[47] They found that troponin-T levels were higher in HF patients compared to healthy control subjects. Furthermore, troponin-T concentration was higher in HFREF than in HFNEF, even after adjusting for clinical covariates, which suggests that myocyte injury is higher in HFREF.

ST2

ST2 is a member of the interleukin-1 receptor family and is involved in the process of ventricular remodeling.[47] ST2 may be upregulated in cardiac myocytes and fibroblasts subjected to mechanical stress. Santhanakrishnan et al. found that HFNEF patients had higher serum levels of ST2 compared to healthy control subjects.[47] However, this difference did not remain statistically significant after adjustment for age, sex and clinical covariates. Furthermore, there was no difference in ST2 concentration between HFREF and HFNEF patients. Nevertheless, previous studies have demonstrated that ST2 was an independent predictor of mortality in patients with acute HF and that ST2 was equally predictive in patients with HFREF and HFNEF.[54]

DISCUSSION

Although a significant body of research has been generated in the past decade on the role of biomarkers in HF, the majority of prognostic studies have included HFREF patients. Studies on the prognostic and incremental value of biomarkers, other than BNP and NT-proBNP, in HFNEF are scarce or lacking. Most of the studies on biomarkers in HFNEF patients are cross-sectional in design and have included limited numbers of patients. Only a few prospective studies have been conducted. To the best of our knowledge, this is the first review that specifically focuses on the role of biomarkers in HFNEF.

Biomarkers examined most extensively in HFNEF include biomarkers of myocyte stress, inflammation and extra-cellular matrix remodeling. Some of these biomarkers have been shown to be increased to a different extent in HFNEF and HFREF.[19, 28, 42] In general, the degree of marker expression in the failing myocardium is likely to depend on the type, degree, and duration of the specific extracellular stimuli. As described above, inflammatory biomarkers may have different meanings in HFNEF and HFREF. Obesity and the metabolic syndrome, associated with increased concentration of several inflammatory and metabolic markers, may drive development of HF, particularly in HFNEF.[24] On the other hand, inflammatory biomarkers may be a measure of severity of HF, particularly in HFREF.[23] Furthermore, fibrosis and myocardial stiffness occur to a different extent and have a different nature in HFNEF versus HFREF.[34] In HFNEF, myocyte stiffness may play a more important role than myocardial fibrosis.[37] This may be reflected by differences in levels of biomarkers of extracellular-matrix remodeling between HFNEF and HFREF.

Specifically, HFREF, typically characterized by volume overload, left ventricular dilatation, eccentric left ventricular remodeling and low relative wall thickness, can particularly be expected to display upregulation of biomarkers such as NT-proBNP (myocyte stress), GDF-15 (stress pathway), TNF-α (stimulating MMP expression), several MMPs (proteolytic enzymes) and CITP (collagen degradation).[28, 46] On the other hand, HFNEF, characterized more often

by a non-dilated left ventricle, concentric left ventricular hypertrophy and probably driven by metabolic syndrome, may particularly be associated with upregulation of biomarkers such as TIMPs (inhibiting collagen proteolysis), Galectin-3 (fibroblast activation), PINP, PIIINP (collagen biosynthesis), homocysteine (associated with hypertrophy) and resistin (adipose tissue and insulin resistance).[15, 21, 50] In this review, we have observed that levels of several biomarkers of inflammation, including CRP, PTX-3, TNF-α, IL-6 and IL-10, are higher in HFREF compared to HFNEF, although some of these results were not consistent between studies. Together with previous data on MMP and TIMP levels in several animal models, these observations provide some support for the above-mentioned hypothesis regarding the differences between biomarker patterns in HFNEF and HFREF.

Based on the present review, several biomarkers and biomarker categories, including biomarkers of myocyte stress, inflammation, extracellular matrix remodeling, GDF-15, cystatin C, resistin and galectin-3, appear to be potentially promising diagnostic tools in HFNEF. Some of them, including TNF-α, IL-6, PINP, PIIINP, osteopontin and cystatin C, may carry prognostic value as well. Further research may provide additional evidence for the value of these biomarkers in improvement of risk stratification of patients with HFNEF. Furthermore, the balance between various markers wintin the same category (e.g. MMP/TIMP ratios and balance between inhibitory and stimulatory cytokines) may also have diagnostic and prognostic value, and should be further investigated as well. Applying a multiple-biomarker strategy may result in even further improvement of risk stratification compared to using one biomarker alone.[15, 30] For example, a multiple-biomarker panel consisting of increased MMP-2, TIMP-4, PIIINP, and decreased MMP-8 was able to identify HFNEF patients with an area under the receiver operating characteristic curve of 0.79, which was better than any single biomarker, including NT-proBNP, or clinical covariates alone.[30] Such results are promising and should be further investigated before such biomarkers can be used in clinical practice.

Some of the studies we reviewed displayed inconsistent results. This may in part be explained by the lack of power to detect significant differences in biomarkers levels and clinical outcomes, as sample size was modest in many of the studies. Furthermore, various definitions of HFNEF were used by the individual studies, and the LVEF cutoff value ranged from ≥40% to ≥55%. Moreover, large variations were present in the choice of the reference groups.

Future directions

Currently, natriuretic peptides are the only biomarkers routinely used for diagnosis and risk stratification in common clinical practice.[1, 2] Before other known biomarkers may be used, their incremental diagnostic and prognostic value over established clinical covariates and imaging techniques, such as echocardiography, should be evaluated. Preferably, this should

be done in an epidemiological setting using a gold standard, including measures of cardiac structure and function in addition to clinical presentation, to demonstrate the diagnostic power of a specific biomarker. Moreover, as mentioned above, a multiple-biomarker panel may have higher diagnostic and prognostic value than any single biomarker alone. Therefore, large, prospective studies measuring multiple biomarkers are urgently needed in order to further elucidate the role and value of biomarkers in HF in general. With regard to the diagnostic and prognostic value of biomarkers in HFNEF in particular, evidence is much less abundant so far and studies have again mainly focused on natriuretic peptides. The other, most promising, biomarkers pertaining specifically to HFNEF at the moment include markers of collagen turnover and collagen signaling pathways.

Meanwhile, it is likely that ongoing fundamental and epidemiologic research will also yield new classes of potentially useful HF biomarkers. Several, relatively novel research techniques, such as genomics and proteomics, are promising contributors to biomarker discovery. New biomarkers may contribute to further improvement of prognostication and may improve our understanding of the complex pathophysiology of HF as well. Moreover, by comparing biomarker patterns and their prognostic value between patients with reduced and normal LVEF, further etiologic insights into the development of HFNEF may be obtained.

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71

Blood biomarkers in patients who received a donor heart

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Blood biomarkers in patients
who received a donor heart

7.1|

Blood biomarkers for diagnosis of acute allograft rejection

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Submitted

ABSTRACT

Introduction: Several studies have examined the diagnostic value of blood biomarkers for acute allograft rejection (AR) after heart transplantation (HTx). However, most of them examined only one biomarker and results were generally inconsistent. The aim of our study was to investigate the diagnostic value of NT-proBNP, troponin T (TropT) and CRP levels for concomitant AR during the first year after HTx.

Methods: From 2005 to 2010, 77 consecutive HTx recipients were included. NT-proBNP, TropT and CRP were measured at 16±4 (mean±SD) consecutive routine endomyocardial biopsy (EMB) surveillance visits during the first year of follow-up. AR was defined as ISHLT grade ≥ 2R at EMB. Data were analyzed using generalized estimating equations (GEE) and area under the receiver-operating characteristic (ROC) curves (AUCs).

Results: Median age of the patients at HTx was 49 years, and 68% were men. During the first year of follow up, 56 patients (73%) experienced at least one episode of AR. The temporal evolution of NT-proBNP, troponin T and CRP showed a steep decline in the first 3 months after HTx and a steady-state level thereafter. CRP level was associated with concomitant AR by means of a threshold effect (odds ratio(OR) and 95% CI, 2.20(1.41-3.44), p=0.001, for the middle vs lowest, and 1.61(1.00-2.60), p=0.050, for the highest vs lowest tertile of CRP). Adding CRP to a model containing the clinical variables age, gender, diabetes, hypertension, and smoking (AUC= 0.64) resulted in higher discriminatory ability during the steady state period, although the difference was not statistically significant (AUC= 0.70, p=0.25). NT-proBNP and troponin T were not significantly associated with AR.

Conclusions: CRP level carries limited diagnostic value for concomitant presence of AR in the first year after HTx. Discriminative ability of CRP is too poor to replace endomyocardial biopsy.

INTRODUCTION

Acute allograft rejection (AR) is one of the major causes of cardiac graft loss after heart transplantation (HTx).(1) While endomyocardial biopsy (EMB) is currently the gold standard for diagnosing allograft rejection in heart transplant recipients, this is an invasive procedure that carries potential complications.(2) Non-invasive detection of allograft rejection would decrease patient discomfort, lower risk of complications and decrease healthcare costs. Although earlier studies suggest a role for biomarkers in the noninvasive diagnosis of AR (3-8), the results of these studies are inconsistent.

NT-pro-B-type natriuretic peptide (NT-proBNP), cardiac troponin T and C-reactive protein (CRP) are biomarkers which may be expected to be released during allograft rejection. These biomarkers reflect several pathophysiological mechanisms that may play a part in allograft rejection, and thus the information carried by these biomarkers is complementary. NT-proBNP represents systolic and diastolic dysfunction which is induced by allograft rejection to some degree, (9) troponin T reflects cardiac myocyte damage, which is the hallmark of moderate to severe allograft rejection,(10) and CRP represents the proinflammatory state which exists in allograft rejection (i.e., noninfectious myocarditis).

Existing studies on the value of NT-proBNP, troponin T, or CRP for diagnosis of allograft rejection have provided inconsistent results, in part due to differences in methodology. (3-8, 11-21) Of further note is that the methodology applied by these studies was not always appropriate. Transplant recipients generally undergo a large number of repeated endomyocardial biopsies according to a standard protocol. Most of the studies examined biomarker measurements performed concomitantly with these EMBs, and thus contained multiple biomarker measurements and biopsy specimens per patient. However, several studies either failed to describe how they accounted for dependence among repeated observations in individual patients, or failed to account for this dependence altogether.

The main objective of this study was to examine the diagnostic value of NT-proBNP, troponin T, and CRP for concomitant allograft rejection in heart transplant recipients. We aimed to account for correlated data within individual patients in the analysis by using appropriate statistical methods.

METHODS

Study population and baseline characteristics

We included a total of 77 consecutive patients, 18 years of age and older, which successfully underwent HTx at the Erasmus MC, Rotterdam, The Netherlands, between September 2005 and December 2010. Patients who did not survive the HTx procedure were not included in this cohort. From 2005 onwards, we performed routine measurements of NT-proBNP,

CRP and troponin T in the context of usual care in post-HTx patients, concomitant with the routine endomyocardial biopsy surveillance visits at our centre. For the current study, data on these 3 biomarkers were retrieved retrospectively from the medical records, and these data were complete in 95, 98 and 96 % of the patients for NT-proBNP, CRP and troponin T, respectively. Baseline characteristics were collected from the heart transplantation screening records and included diabetes (known history of diabetes or use of antidiabetic agents), hypertension (known history of hypertension), hypercholesterolemia (known history of hypercholesterolemia), body mass index (BMI) (kg/m2), smoking status (never smokers or former smokers (> 1 month)), and indication for transplantation (ischemic or non ischemic). Ischemic time and use of immunosuppressive medication were collected from the discharge letters and medical records.
This study was approved by the ethical committee of the Erasmus MC. According to Dutch law, informed consent was not required, since study specific actions were not implemented. All data were readily available in the (electronical and paper-based) medical records of the patients and were obtained during routine treatment. Subsequently, data were processed in an anonymized manner.

Biomarker measurement

Concomitant with the endomyocardial biopsy, blood was drawn from the intravascular catheter, which was generally placed in the vena jugularis interna dextra.. Plasma NT–pro-BNP was measured using an electrochemiluminesence assay (Roche Diagnostics, Elecsys 2005, Indianapolis, Indiana, USA). This system measures concentrations ranging 0.6 to 4130 pmol/L. The assay had a total coefficient of variation that ranged between 2.3 and 3.2%, depending on mean concentration. Cardiac troponin T was measured using an immunoassay (Roche Diagnostics, Elecsys 2010 immunoassay analyser, Indianapolis, Indiana, USA). This system measures concentrations ranging from 0.01 to 25 ng/mL, with a total precision <10 %. CRP was measured using an immunoturbidimetric assay (Roche Hitachi 912 chemistry analyzer, Basel, Switzerland). This system measures concentrations ranging from 0.3 to 350 mg/L, with a total precision <10 %.

Follow-up

Patients underwent endomyocardial biopsies according to the standard biopsy protocol, which consists of weekly biopsies until week 6, and subsequent biopsies at week 8, 10, 14, 18, 24, 30, 38, 46 and 52. Exceptions were possible based on individual situations. The revised International Society for Heart and Lung Transplantation (ISHLT) grading system was applied to classify the endomyocardial biopsies.(22) Allograft rejection was defined as the presence of an EMB with an ISHLT grade 2R or greater.

Table 1. Baseline characteristics of the 77 heart transplant recipients

Variable	Mean±SD, median [IQR] or No. (%)
Age, years	49 [42-55]
Male gender	52 (68)
Diabetes	15 (20)
Hypertension	11 (14)
Hypercholesterolemia	13 (17)
Body Mass Index (kg/m^2)	24±3.9
Smoking	
Never	58 (75)
Former	19 (25)
Indication for transplantation	
Ischemic cardiomyopathy	31 (40)
Non ischemic cardiomyopathy	46 (60)
Ischemic time, minutes	194±36
Medications	
Tacrolimus	74 (96)
Prednisone	75 (97)
Mycophenolate	67 (87)
Cyclosporine	2 (3)
Everolimus	4 (5)

Statistical analysis

Categorical variables are presented in percentages. We examined the distributions of continuous variables for normality by visual examination of the histogram and with the Kolmogorov-Smirnov test. Normally distributed continuous variables are presented as mean ± standard deviation (SD) while non-normally distributed continuous variables are presented as median and interquartile range (IQR). Distributions of NT-proBNP, troponin T and CRP were skewed and these variables were ln-tranformed for further analyses.

Logistic regression was applied to examine whether baseline levels of NT-proBNP, troponin T or CRP are associated with the number of allograft rejections in the first year post-HTx (i.e., remaining free of rejection versus occurrence of one or more rejections; and occurrence of zero or one versus two or more rejections).

Subsequently, generalized estimation equations (GEE) were applied to investigate whether repeatedly measured NT-proBNP, troponin T or CRP are associated with concomitant allograft rejection in the first year post-HTx. GEE is a method meant for analyzing correlated longitudinal data,(23) and may be applied to modeling binary outcomes such as, in this case, allograft rejection. GEE consists of two parts, namely, the mean model and a working covariance model. The mean model characterizes the mean response and its dependence

on selected covariates.(24) The working covariance model describes the pattern of associations amongst the repeated measurements within each individual ('correlation structure').(24, 25) We used an autoregressive (AR) correlation structure for all three biomarkers. This correlation structure assumes decreasing correlation as the distance between two measurements increases,(24) and thus is appropriate for the current study design.(26) We used repeated biomarker measurements available up to one year post-HTx. Biomarker concentrations were examined as ln-transformed continuous variables in order to evaluate linear trends, and as categorical variables (by dividing the variables into tertiles) in order to evaluate potential threshold effects. Age and gender were included as covariates in the GEE.

We examined incremental discriminative value of adding NT-proBNP, troponin T or CRP to clinical characteristics by calculating area under the receiver operating characteristic curve (AUC). AUCs were compared by Medcalc(27).

SPSS statistics version 20.0 was used to perform the analyses. P-values <0.05 were considered statistically significant.

RESULTS

Descriptives

Baseline characteristics are summarized in Table 1. Median age was 49 years and 68% were men. A total of 1136 biopsies and concurrent blood samples were obtained at different time points from the 77 patients. The mean number of measurements was 16 ± 4 (mean±SD), and the mean follow-up time was 320 days. During the first year of follow up, 56 patients (73%) experienced at least one episode of allograft rejection. The total number of rejection episodes in the first year was 121. The numbers of rejection episodes are displayed in Figure 1. A total of 21 patients remained free of allograft rejection in the first year post HTx. Most patients (n= 23) experienced 1 rejection episode. Fewer patients experienced 2 or more rejection episodes (figure 1). The number of patients who experienced a rejection episode for each week number is displayed in figure 2. Most allograft rejections occurred in the first 6 months post-HTx, with a peak in week 3 (16 allograft rejections).

Baseline biomarker levels and number of allograft rejections during first year post-HTx

The associations of NT-proBNP, troponin T and CRP with the number of AR episodes are displayed in tables 2a and 2b. No associations were found between any of the three biomarkers and occurrence of one or more AR episodes during the first year post HTx (table 2a). Similar results were found for patients with zero or one AR episode versus patients who experienced two or more AR episodes (table 2b).

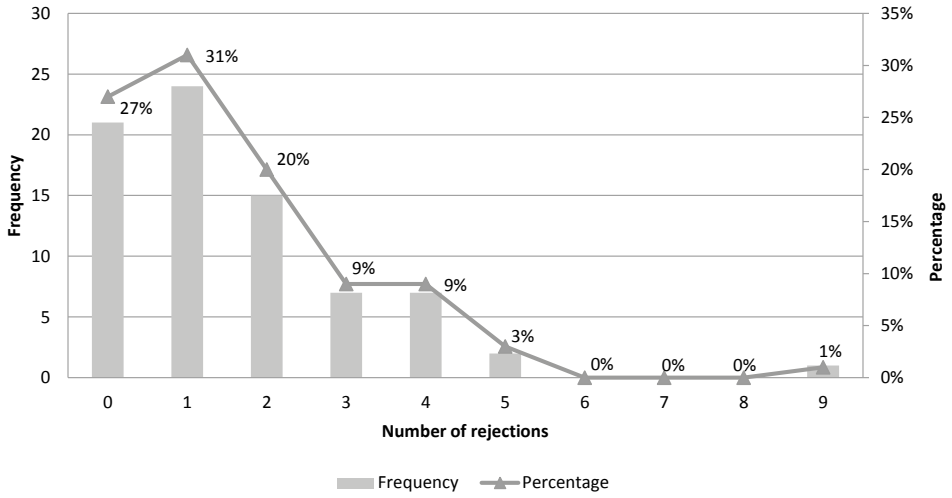


Figure 1. The number of rejection episodes during the first year post-HTx.

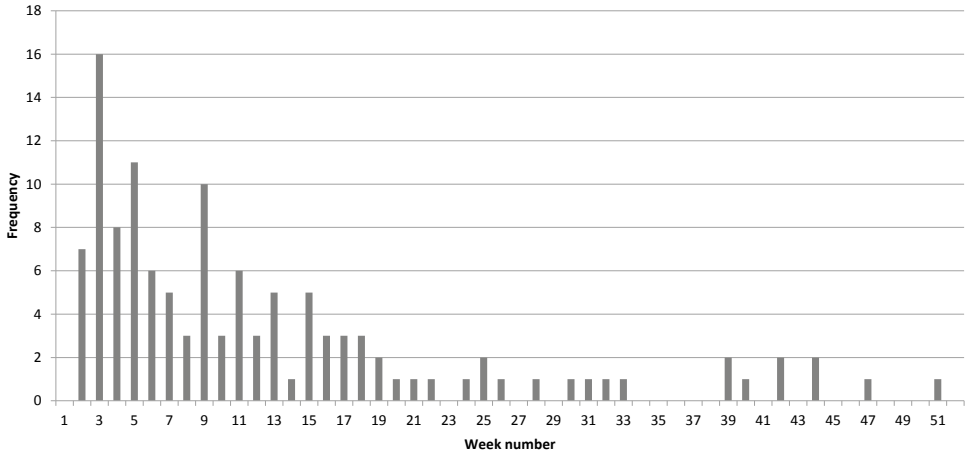


Figure 2. Number of patients who experienced a rejection episode for each week number during the first year post-HTx.

Temporal evolution of the biomarkers

The temporal evolutions of NT-proBNP, troponin T and CRP, showed a steep decline in approximately the first 3 months after HTx and a steady-state level thereafter (figure 3 a, b and c). The biomarker values over time in patients who experienced a rejection episode during a particular visit did not show clear differences compared to patients who did not experience a rejection episode during that same visit (figure 4 a, b, c, d, e, and f).

Table 2 a. Association of baseline (post-HTx) NT-proBNP, troponin T, and CRP levels with the occurrence of acute allograft rejection episodes during the first year post-HTx.

Any acute AR (vs no acute AR)	Unadjusted model		Multivariable model*	
	Odds ratio (95%CI)	P	Odds ratio (95%CI)	P
NT-proBNP#	0.93 (0.84-1.03)	0.17	0.93 (0.84-1.03)	0.18
Troponin T#	0.95 (0.84-1.08)	0.42	0.94 (0.83-1.07)	0.39
CRP#	0.98 (0.89-1.08)	0.68	0.98 (0.89-1.08)	0.75

*adjusted for age and gender; # Ln transformed; AR=allograft rejection

Table 2 b. Association of baseline (post-HTx) NT-proBNP, troponin T, and CRP levels with the occurrence of two or more acute allograft rejection episodes during the first year post-HTx.

Two or more acute ARs (vs zero or one acute AR)	Unadjusted model		Multivariable model*	
	Odds ratio (95%CI)	P	Odds ratio (95%CI)	P
NT-proBNP#	0.90 (0.81-1.02)	0.093	0.90 (0.80- 1.00)	0.047
Troponin T#	0.91 (0.79-1.05)	0.20	0.92 (0.80-1.07)	0.28
CRP#	0.99 (0.89-1.12)	0.94	0.99 (0.89-1.11)	0.88

*adjusted for age and gender; # Ln transformed; AR=allograft rejection

Diagnostic value of biomarkers for concomitant allograft rejection

Given the observed steep decline of the three biomarkers in the first three months post-HTx, we corrected all GEE analyses for the time-period in which the biomarkers were determined. After visual examination of figures 3 and 4, we chose 3 months post-HTx as the boundary between the period of 'steep decline' and the period of 'steady state'. The multivariable analyses were additionally adjusted for age and gender. The results are shown in table 3. NT-proBNP and troponin T were not associated with allograft rejection: p for linear association (ln transformed, continuous biomarkers) was not significant for these biomarkers, and analysis of biomarker tertiles did not display statistically significant threshold effects. For CRP, higher levels were associated with AR, with a threshold effect for CRP values higher than tertile 1 (i.e., 1 mg/L). The age- and sex adjusted ORs and 95% CIs for the presence of allograft rejection were 2.20 (1.41-3.44), p=0.001, for the middle vs the lowest tertile of CRP, and 1.61 (1.00-2.60), p=0.050, for the highest vs the lowest tertile of CRP. Again, p for linear association was not significant. The OR for the 'steady state' time period was smaller than 1 in all biomarker models, indicating that the odds of having an AR episode after 3 months post-HTx is smaller than the odds of having an AR episode in the first 3 months post-HTx. This was in line with our descriptives (figure 2). No interaction was present between any of the biomarkers and time period, indicating that associations

between the biomarkers and AR was similar in months 0-3 and months 4-12 post-HTx, in spite of the steep decline in levels (age- and sex adjusted ORs (95%CIs) of interaction term 'time period'*'ln (biomarker)'were 0.79 (0.51-1.22), p=0.47, 0.91 (0.70-1.18), p=0.46, and 1.11 (0.85-1.45), p=0.44, for NT-proBNP, troponin T and CRP, respectively). When we repeated the analysis using data from the steady state period (4-12 months) only, results did not change materially (data not shown).

Table 3. Odds ratios for allograft rejection during week 0-53 post HTx

Variable	OR (95% CI) Period** adjusted	P-value	OR (95% CI) Period-, age- and sex adjusted	P-value
NT-proBNP				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.99 (0.59-1.66)	0.96	0.99 (0.59-1.67)	0.97
Tertile 3	0.93 (0.51-1.70)	0.82	0.94 (0.51-1.72)	0.83
NT-proBNP#	1.15 (0.92-1.42)	0.22	1.15 (0.93-1.43)	0.21
Steady state period	0.53 (0.33-0.84)	0.007	0.53 (0.33-0.84)	0.008
Troponin T				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.61 (0.37-1.01)	0.055	0.61 (0.37-1.00)	0.051
Tertile 3	0.74 (0.45-1.21)	0.23	0.74 (0.45-1.22)	0.24
Troponin T#	0.99 (0.86-1.13)	0.86	0.99 (0.86-1.14)	0.90
Steady state period	0.53 (0.33-0.84)	0.007	0.53 (0.33-0.84)	0.008
CRP				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	2.18 (1.39-3.42)	0.001	2.20 (1.41-3.44)	0.001
Tertile 3	1.60 (0.99-2.58)	0.057	1.61 (1.00-2.60)	0.050
CRP#	1.09 (0.97-1.23)	0.14	1.10 (0.98-1.23)	0.12
Steady state period	0.53 (0.33-0.84)	0.007	0.53 (0.33-0.84)	0.008

OR (95% CI)= odds ratio, 95% confidence interval; **Period= steady state (4-12 months) versus steep decline (0-3 months); # Ln transformed

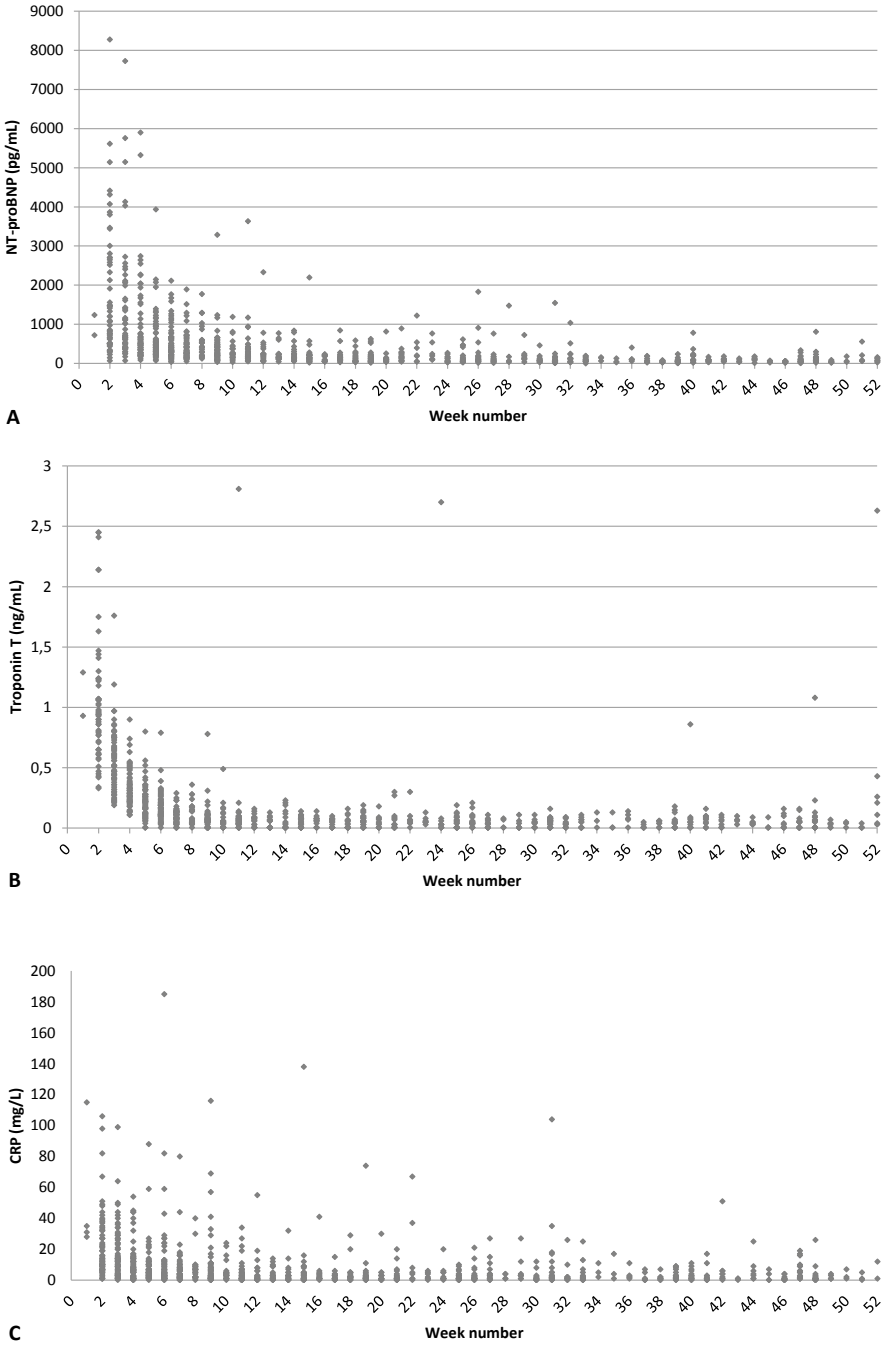


Figure 3 a, b and c. Absolute values of NT-proBNP, troponin T and CRP, for each week number, during the first year post-HTx.

Univariable ORs for the available clinical characteristics in relation to allograft rejection are displayed in table 4. None of the clinical characteristics we had registered was associated with allograft rejection occurring over the entire follow-up period, i.e. month 0-12 post HTx. Conversely, in the steady state period of 4-12 months follow-up, ORs for diabetes (3.25 (95%CI 0.87-12.08)) and hypertension (2.84 (95%CI 0.59-13.62)) showed to be higher, although not statistically significant. The combination of the clinical characteristics age, gender, diabetes, hypertension, and smoking displayed the highest discriminatory value, with an AUC of 0.61 over the total follow-up period. Adding CRP to these clinical characteristics resulted in slightly higher discriminatory ability, although the difference was not statistically significant (AUC = 0.63, p = 0.64) (figure 5). Adding NT-proBNP and troponin T to the clinical characteristics did not provide any incremental value to the clinical characteristics. For the steady state period of 4-12 months of follow-up, the AUC for this combination of clinical characteristics was 0.64. Adding CRP resulted in somewhat higher discriminatory ability, although again the difference was not statistically significant (AUC = 0.70, p = 0.25) (figure 5). Again, adding NT-proBNP and troponin T to the clinical characteristics did not provide any incremental value.

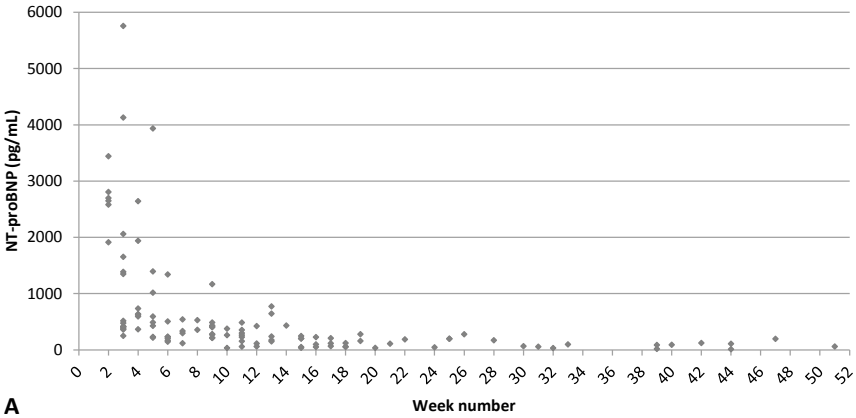
Table 4. Univariable associations of clinical characteristics with presence of AR

Variable	OR (95% CI) (0-12 months follow-up)	P-value	OR (95% CI) (4-12 months follow-up)	P-value
Age	0.99 (0.97-1.02)	0.58	1.01 (0.97-1.05)	0.55
Gender	0.86 (0.54-1.36)	0.52	0.73 (0.34-1.59)	0.43
Diabetes	0.66 (0.36-1.21)	0.18	3.25 (0.87-12.08)	0.079
Hypertension	0.66 (0.30-1.47)	0.31	2.84 (0.59-13.62)	0.19
Hypercholesterolemia	0.57 (0.28-1.18)	0.13	0.74 (0.27-1.99)	0.55
Smoking	1.28 (0.85-1.94)	0.23	0.65 (0.31-1.37)	0.26

OR (95% CI)= odds ratio, 95% confidence interval

DISCUSSION

In this study we investigated the diagnostic potential of NT-proBNP, troponin T and CRP to detect acute allograft rejection in the first year post-HTx in 77 heart transplant recipients. We found that biomarker concentrations were elevated directly after HTx, decreased during the first three months after HTx, and stabilized thereafter. No associations were found between baseline biomarker levels and number of acute allograft rejection in the first year post-HTx. When examining repeated biomarker measurements, we found an association of CRP level higher than tertile one (i.e., 1 mg/L) and occurrence of concomitant acute allograft rejection. However, discriminative ability of CRP was too weak to replace endomyocardial biopsy.



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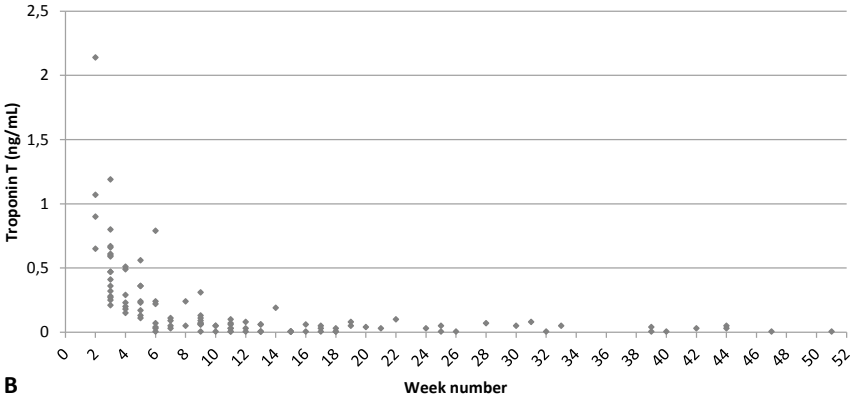

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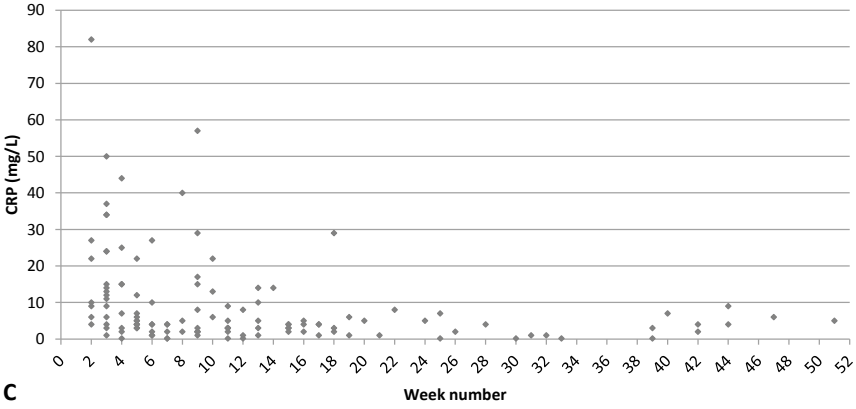

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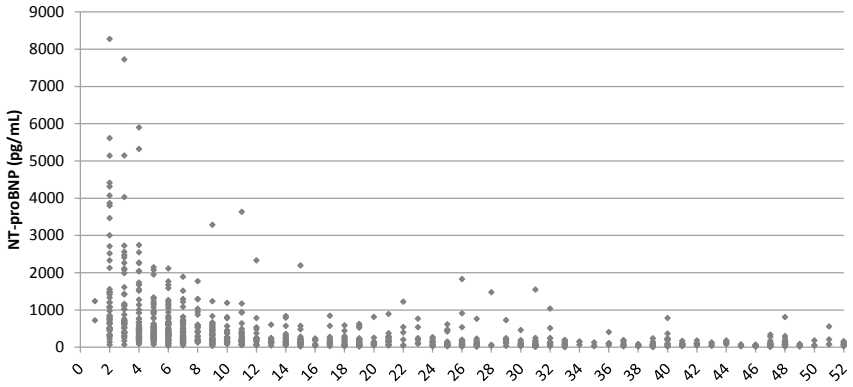
Figure 4 a, b, c, d, e, and f. Absolute values of NT-proBNP, troponin T and CRP, for each week number, in patients with (a, b, and c) and without (d, e, and f) AR, during the first year post-HTx.



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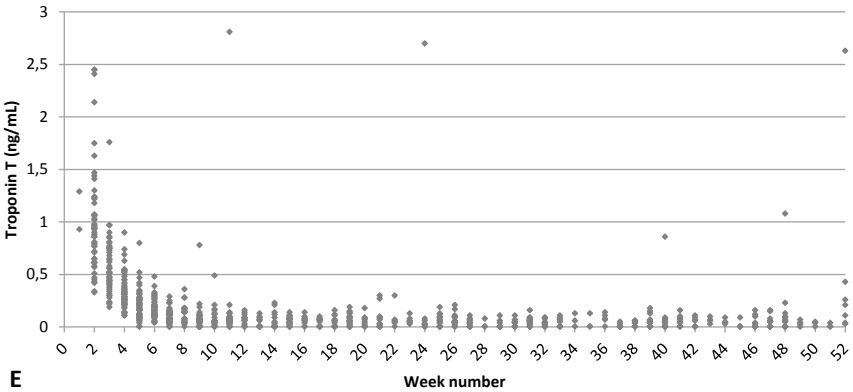

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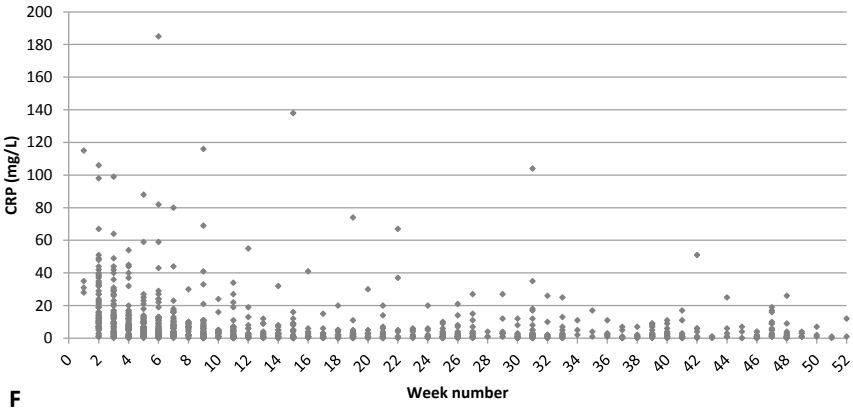

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Figure 4 a, b, c, d, e, and f. Continued.

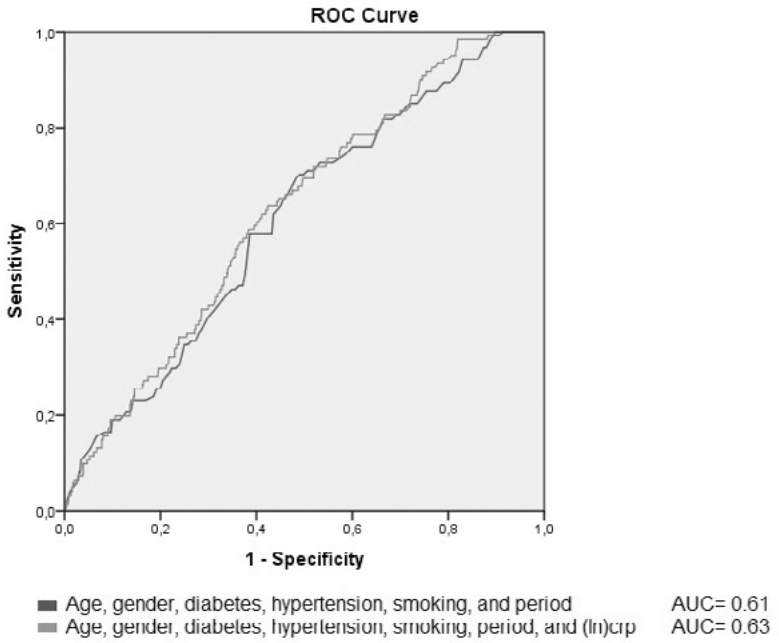

Figure 5. Receiver operating characteristic curve displaying additional discriminatory ability of CRP for AR during time-period 0-12 months and time period 4-12 months

Repeatedly measured NT-proBNP and troponin T did not show any significant associations with concomitant acute rejection. CRP is produced by hepatocytes under the influence of cytokines, including IL-6, and is one of the most sensitive markers of systemic inflammation.(28) Prior studies in renal transplant patients have shown that immunosuppressive therapy with cyclosporine and prednisolone results in suppression of CRP response.(29, 30) Nevertheless, changes in CRP levels over time have been found to correlate with acute rejection in those patients.(30) In contrast, in HTx recipients there is no established consensus about the correlation between CRP levels and acute rejection as diagnosed by EMB.(5, 6, 20) Deng et al.(31) investigated the potential role of monitoring the immunological status in HTx recipients to differentiate between acute rejection and infection in the presence of graft dysfunction. Acute rejection was generally associated with under-immunosuppression, but not with CRP levels, whereas infection was associated with over-immunosuppression and elevated levels of inflammatory markers, including CRP. A recent study of Heikel et al.(32) found the same results and suggested monitoring of immune function may have a potential role for individualization of immunosuppressive therapy and a minimization of unnecessary EMBs. In contrast, Martinez-Dolz et al.(5) found a significant correlation between CRP and acute rejection. However, despite a high

specificity of CRP levels below 0.87 mg/dL, the sensitivity was poor. In line with this, we found a tendency towards an association of elevated levels of CRP with concomitant acute rejection during the first year after HTx, in spite of the fact that part of the patients were on immunosuppressive therapy at the time of blood sampling and EMB. However, with an AUC of 0.70 in the steady state period of follow-up, discriminative value of CRP was poor.
We did not find an association between NT-proBNP levels and acute rejection. Previous studies on the diagnostic value of natriuretic peptides for allograft rejection have rendered inconsistent results (12, 15, 21, 33). Moreover, in many of the studies that did find positive associations, the specificity of BNP for acute rejection was inadequate to replace endomyocardial biopsy.(15, 21) These inconsistent results may in part be due to the fact that a large number of parameters are associated with elevated BNP levels. BNP is secreted by the heart in response to myocardial stretch(33) to protect the organism against pressure and volume overloads, and is generally increased during heart failure. (13),(34) A substantial decline of NT-proBNP in the initial months after HTx has been found in previous studies, (11, 12) however, several studies have shown that these levels do not normalize even years after HTx.(33, 34) These observations suggest that BNP should not be considered solely as a haemodynamic marker, and have generated much discussion about the mechanisms of NT-proBNP release in the transplanted heart. A potential explanation is that BNP may respond to immunological and/or inflammatory stimuli, which may induce increased circulating BNP concentrations secreted by cardiomocytes or other cell types infiltrating the heart.(33, 35) Torre-Amione et al.(36) examined human cardiac allografts, in the absence of histopathological or clinical evidence of rejection and with normal LV systolic function, and found an overexpression of TNF-α. A persistently elevated TNF-α level may stimulate BNP mRNA and BNP secretion, as demonstrated in neonatal rat ventricular cardiocytes.(37) During acute rejection, exacerbation of inflammatory processes, and in particular elevated IL-1β or TNF-α levels, may lead to increased secretion as well as expression of BNP.(38) Furthermore, a decreased renal clearance in the presence of ventricular hypertrophy, systemic or pulmonary hypertension or systolic dysfunction is also associated with increased BNP levels.(13, 33) Altogether, a large spectrum of parameters may influence natriuretic peptide levels, and this might have obscured a potential association of BNP with acute rejection to a certain extent in the current study.
Troponin T and I are cardiospecific myofibrillar proteins, which are released during myocyte damage.(39) Several studies have found a continued release of troponin T over the first three months post HTx,(40, 41) although, the pathofysiology of this release is still not fully elucidated. Troponin T is not solely a cardiac marker since elevated levels are also found in patients with acute pulmonary embolism, sepsis, and end-stage renal disease.(42) In a previous study, Mullen et al.(18) investigated the association of both troponin T and I with acute rejection, but concluded that these markers are not useful as indicators of rejection.

Similarly, in the current study, we were not able to demonstrate a significant association between troponin T and acute rejection. Since the troponin T assay that was available at the time of this study was not as sensitive as the currently available high sensitive (hs)-troponin T assays, minor myocyte damage may have remained undetected. This may in part explain the lack of an association.

Strengths of the present study include the availability and use of a large number of biopsies and the consecutive repeated measurement of 3 biomarkers, which represent various pathophysiological pathways underlying allograft rejection. Furthermore, we have applied appropriate statistical methods, that take into account correlated biomarker measurements within each individual, to examine the diagnostic value of repeated biomarker measurements for concomitant allograft rejection. Limitations of the current study include its retrospective nature. Nevertheless, all data required could be collected from the medical records, given that this patient population is under strict clinical surveillance. Furthermore, troponin T was measured using an assay which is not as sensitive as the currently available hs-troponin T assays.

In conclusion, we found a positive association between CRP level and concomitant acute allograft rejection. However, discriminative ability of CRP was poor and thus unable to replace EMB. NT-proBNP and troponin T did not show significant associations with acute rejection in the current study.

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7.2|

Blood biomarkers for prediction of acute allograft rejection

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ABSTRACT

Introduction: Studies on the prognostic value of serial biomarker assays for future occurrence of allograft rejection (AR) are scarce. We examined whether repeated measurements of NT-pro-B-type natriuretic peptide (NT-proBNP), troponin T (TropT) and C-reactive protein (CRP) predict AR.

Methods: In 2005-2010, 77 consecutive HTx recipients were included. NT-proBNP, TropT and CRP were measured at 16±4 (mean±SD) consecutive routine endomyocardial biopsy (EMB) surveillance visits during the first year of follow-up. AR was defined as ISHLT grade ≥2R at EMB. Joint modeling (JM) was used to assess the association between repeated biomarker measurements and occurrence of future AR. JM accounts for dependence among repeated observations in individual patients.

Results: The mean age of the patients at HTx was 49±9.2 years, and 68% were men. During the first year of follow-up, 1136 biopsies and concurrent blood samples were obtained, and 56 patients (73%) experienced at least one episode of AR. All biomarkers were elevated directly after HTx and achieved steady-state after ~12 weeks, both in patients with or without AR. No associations were present between the repeated measurements of NT-proBNP, TropT or CRP and AR both early (weeks 0-12) and late (weeks 13-52) in the course after HTx (hazard ratios for weeks 13-52: 0.96 (95% CI 0.55-1.68), 0.67 (0.27-1.69), and 1.44 (0.90-2.30), respectively, per ln(unit)). Combining the 3 biomarkers in one model also rendered null results.

Conclusions: The temporal evolution of NT-proBNP, TropT and CRP prior to AR did not predict occurrence of acute AR both in the early and late course of the first year post-HTx.

INTRODUCTION

Acute allograft rejection (AR) is a common problem after heart transplantation (HTx) and is one of the major causes of cardiac graft loss.(1) The gold standard for diagnosing AR in heart transplant recipients is endomyocardial biopsy (EMB). This is an invasive procedure that carries potential complications and merely confirms the presence of AR.(2) As a result, treatment can only start at the moment that rejection is already ongoing. Prognostic tools that enable risk stratification of cardiac allograft recipients in an earlier phase could potentially facilitate earlier use of therapeutic measures. Unfortunately, such tools are currently not available.

Blood markers of cardiac stress and malfunction may aid in early, noninvasive detection of AR, and thus are potential candidates for prognostication of cardiac allograft recipients. NT-pro-B-type natriuretic peptide (NT-proBNP) is expected to increase during AR, since it is produced in response to increased ventricular pressure, and AR is known to induce some degree of systolic and diastolic dysfunction.(3) Cardiac troponin T is a cardiospecific myofibrillar protein, which is detectable in the plasma after any cardiac myocyte damage. Since myocyte damage is the hallmark of moderate to severe AR, troponin T might also be expected to be released during significant AR.(4) Furthermore, a proinflammatory state exists in AR (i.e. noninfectious myocarditis) that could be assessed by determining plasma levels of inflammatory markers. C-reactive protein (CRP) is an acute phase protein produced predominantly by hepatocytes, that rises quickly during inflammation.(5, 6)

Previous studies on biomarkers in relation to AR have mostly focused on their diagnostic value and have examined measurements concurrent to the EMB, which already displayed AR. These studies have provided inconsistent results for all 3 biomarkers, in part due to differences in methodology. Fewer studies have examined the prognostic value of the biomarkers. Positive(7, 8), as well as negative(9) associations were found between (NT-pro)BNP and future AR. Studies on CRP and future AR also showed contradicting results. (9, 10) All of these studies have examined the value of baseline biomarker measurements or the change between two measurements. Since biomarker levels change continuously, such approaches do not fully capture the prognostic value of a large number of repeated biomarker measurements.

The main objective of this study was to examine the natural history of NT-proBNP, Troponin T, and CRP after HTx and to investigate the prognostic value of repeated measurements of these biomarkers for the occurrence of allograft rejection in heart transplant recipients. We hypothesized that joint modeling (JM) could potentially better capture prognostic information carried by these biomarkers than simpler statistical models used until now. JM uses the total number of repeated measurements performed before allograft rejection occurs, and accounts for dependence among repeated observations in individual patients. (11)

MATERIALS AND METHODS

Study population and baseline characteristics

We included a total of 77 consecutive patients, 18 years of age and older, which successfully underwent HTx at the Erasmus MC, Rotterdam, The Netherlands, between September 2005 and December 2010. Patients who did not survive the procedure were not included in this cohort. From 2005 onwards, we performed routine measurements of NT-proBNP, CRP and troponin T in the context of usual care in post-HTx patients concomitant with the routine EMB surveillance visits at our centre. For the current study, data on these 3 biomarkers were retrieved retrospectively from the medical records. Baseline characteristics were collected from the heart transplantation screening records and included diabetes (known history of diabetes or use of antidiabetic agents), hypertension (known history of hypertension), hypercholesterolemia (known history of hypercholesterolemia), body mass index (BMI) (kg/m2), and indication for transplantation (ischemic or non ischemic). Ischemic time and use of immunosuppressive medication were collected from the discharge letters and medical records.

This retrospective study on anonymized data was approved by the ethical committee of the Erasmus MC and does not require informed consent according to Dutch laws ('WMO'). No additional actions involving the study participants were undertaken because of this retrospective study.

Biomarker measurement

Concomitant with the EMB, blood was drawn from the intravascular catheter, which was generally placed in the vena jugularis interna dextra. Plasma NT–pro-BNP was measured using an electrochemiluminesence assay (Roche Diagnostics, Elecsys 2005, Indianapolis, Indiana, USA). This system measures concentrations ranging from 0.6 to 4130 pmol/L. The assay had a total coefficient of variation that ranged between 2.3 and 3.2%, depending on mean concentration. Cardiac Troponin T was measured using an immunoassay (Roche Diagnostics, Elecsys 2010 immunoassay analyser, Indianapolis, Indiana, USA). This system measures concentrations ranging from 0.01 to 25 ng/mL, with a total precision <10 %. CRP was measured using an immunoturbidimetric assay (Roche Hitachi 912 chemistry analyzer, Basel, Switzerland). This system measures concentrations ranging from 0.3 to 350 mg/L, with a total precision <10 %.

Follow-up

Patients underwent endomyocardial biopsies according to the standard biopsy protocol, which consists of weekly biopsies until week 6 and subsequent biopsies at week 8, 10, 14, 18, 24, 30, 38, 46 and 52. The revised International Society for Heart and Lung

Transplantation (ISHLT) grading system was applied to classify the endomyocardial biopsies. (6) AR was defined as the presence of an EMB with an ISHLT grade 2R or greater. Patients that experienced allograft rejection before a second blood sample could be collected (n=9) were excluded from further analyses, since they had no repeated biomarker measurements available.

Statistical analysis

JM is a modeling framework which combines mixed models (in our case for serial biomarker measurements) with Cox proportional hazards models (in our case for the risk of AR), and is used to produce valid measures of associations between the two outcomes while accounting for dependence among repeated observations in an individual patient.(11) By combining mixed models with Cox models, follow-up time until occurrence of the event of interest (AR) or until censoring is taken into account, and thus a hazard ratio is computed that represents the association between each of the (repeatedly measured) biomarkers and the outcome of interest. Fields of medical research that have previously applied this method include Human Immunodeficiency Virus research (repeated CD4 cell counts) and prostate cancer studies (repeated prostate specific antigen measurements).(12, 13)

We examined the associations of each of the 3 biomarkers with first AR. While constructing the mixed models, we first tested whether the residuals were normally distributed with a constant variance, and when deemed necessary, we applied transformations of the biomarkers (e.g., taking natural logarithms). We also tested whether quadratic terms improved the mixed models by using Akaike's Information Criterion (AIC) and selected the models with the lowest AIC for further use. We used all repeated biomarker measurements available up to the occurrence of AR. Subsequently, we combined the mixed models with Cox proportional hazards models ('JM'). Herewith, we obtained hazard ratios for the associations of the repeated biomarker measurements with AR. We adjusted the models for age and sex. Adjustment for additional confounders was not possible due to the small number of events.

JM only allows for one determinant in the model to be repeatedly measured. In order to assess the prognostic value of all 3 repeatedly measured biomarkers simultaneously, we standardized the biomarkers and combined them into one variable, which was subsequently used as the determinant in the JM.

For standardization, clinical upper reference limits of the biomarker assays were used (14 pg/mL, 0.02 ng/mL and 9 mg/L for NT-proBNP, Troponin T and CRP, respectively). Each biomarker was expressed as a multiple of the doubling relative to its upper reference value (for example, for NT-proBNP, a level of 14 pg/mL was coded as 1, a level of 28 pg/mL was coded as 2, a level of 56 pg/mL was coded as 3, etc.). Thus, the right-skewed distribution of the biomarkers was accounted for. The 3 re-coded biomarkers were subsequently

combined in 2 ways. First, to investigate the presence of an additive effect, they were added up. Second, to investigate the presence of a multiplicative effect, they were multiplied. R version 2.15.1 was used to perform the analysis, using packages JM (14) and survival.

RESULTS

Baseline characteristics are summarized in Table 1. The mean age of the study population was 49 years, and of those 68% were men. A total of 1136 biopsies and concurrent blood samples were obtained at different time points from the 77 patients. The average number of measurements was 16 ± 4 (mean±SD), during a mean follow-up time of 320 days. During the first year of follow up, 56 patients (73%) experienced at least one episode of AR. Mean follow-up time until the first episode of AR was 48 days. Figure 1 shows the number of patients, per number of AR episodes experienced, during the first year post-HTx. Furthermore, a total of 5 (6%) patients died during the first year post-HTx and the date of death was used for censoring patients when examining allograft rejection as the endpoint.

Table 1. Baseline characteristics of the 77 heart transplant recipients

Variable	Mean±SD, or No. (%)
Age, years	49±9.2
Male gender	52 (68)
Diabetes	15 (20)
Hypertension	11 (14)
Hypercholesterolemia	13 (17)
Body Mass Index (kg/m^2)	24±3.9
Indication for transplantation	
Ischemic cardiomyopathy	31 (40)
Non ischemic cardiomyopathy	46 (60)
Ischemic time, minutes	194±36
Immunosuppressive regime	
Tacrolimus	74 (96)
Prednisone	75 (97)
Mycophenolate	67 (87)
Cyclosporine	2 (3)
Everolimus	4 (5)

Temporal evolution of the biomarkers

At the time of the first routine EMB surveillance visit (week 1), median NT-proBNP, Troponin T and CRP was 1081 [507-2702] pg/mL, 0.9 [0.6-1.2] ng/mL, and 14 [8-31] mg/L, respectively. The temporal evolution of all three biomarkers, as evaluated by mixed models, showed a steep decline in approximately the first 12 weeks after HTx and a steady-state level thereafter (figure 2 panel a,b and c). For this reason, we divided the total follow-up duration into two time-windows, namely 0-12 weeks and 13-52 weeks, for the remaining analyses.

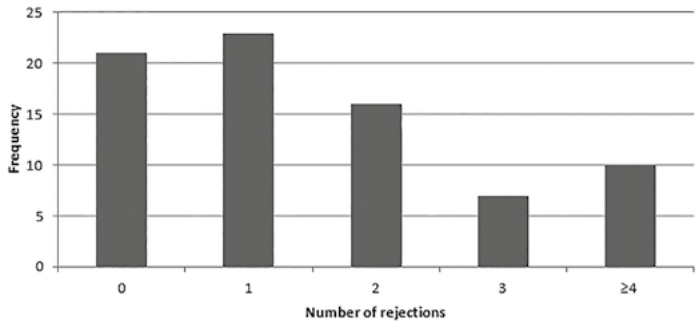
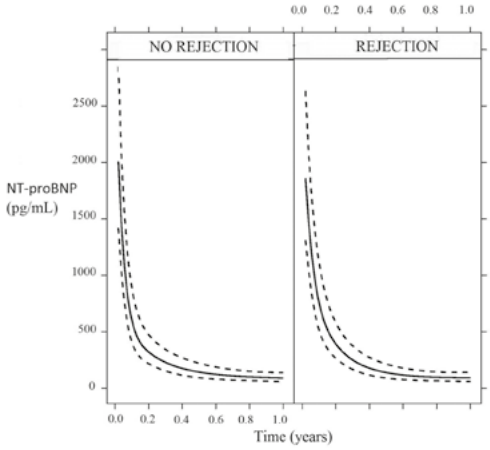


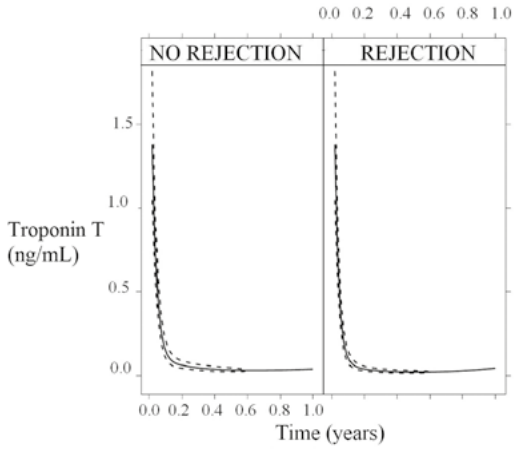
Figure 1. The number of allograft rejection episodes experienced in the first year post-HTx.

Allograft rejection

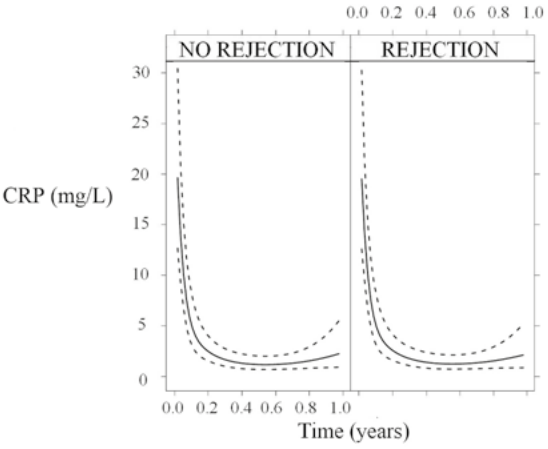
The results of the analyses for the individual biomarkers for weeks 0-12 are shown in Table 2. Of note is that JM only takes into account the first AR that occurs during the time window of interest (in this case, 0-12 weeks) even if a patient experienced additional AR episodes later in time.During weeks 0-12, 47 (61% of 77 patients) 'first' AR episodes occurred. Not all patients who experienced AR could be taken into account in the JM analyses, since some patients had only one biomarker measurement available. These patients experienced an early AR episode before a second blood sample could be collected during follow up (n= 9 for NT-proBNP, Troponin T, and CRP), or had missing values due to miscellaneous reasons resulting in only one available biomarker measurement (n= 5, 5, and 1 for NT-proBNP, Troponin T, and CRP, respectively). Consequently, of the 77 HTx patients, the total numbers of patients available for the JM analyses were 63 (82%), 63 (82%), and 67 (87%) for NT-proBNP, Troponin T, and CRP, respectively. Age and sex adjusted hazard ratios for developing AR obtained with JM were 1.30 (95% CI 0.91-1.86), 1.03 (0.65-1.63), and 1.25 (0.91-1.70) per ln(unit)), for NT-proBNP, Troponin T and CRP, respectively.



Panel A



Panel B



Panel C

Figure 2. Temporal evolution of NT-proBNP, Troponin T, and CRP levels in patients without and with rejection during the first year after HTx**.**

Table 2. Hazard ratios for allograft rejection during weeks 0-12 and weeks 13-52 post-HTx

	Hazard ratio (95% confidence interval)	
Variable	**Week 0-12 post-HTx**	**Week 13-52 post-HTx**
NT-proBNP*	1.30 (0.91-1.86)	0.96 (0.55-1.68)
Age	0.98 (0.95-1.01)	1.00 (0.95-1.05)
Gender	0.77 (0.41-1.45)	1.17 (0.48-2.83)
Troponin T*	1.03 (0.65-1.63)	0.67 (0.27-1.69)
Age	0.98 (0.95-1.01)	1.00 (0.96-1.05)
Gender	0.72 (0.38-1.35)	1.16 (0.48-2.81)
CRP*	1.25 (0.91-1.70)	1.44 (0.90-2.30)
Age	0.98 (0.95-1.01)	0.98 (0.93-1.03)
Gender	0.67 (0.37-1.23)	1.22 (0.50-2.96)

* Ln transformed

The results of the analyses for the individual biomarkers for weeks 13-52 are also shown in Table 2. During this time-period, 27 (35% of 77) "first" AR episodes occurred. Some of these "first" AR (n=19) were in fact *recurrent* ARs in persons that had already experienced AR in weeks 0-12. The total number of available patients for the JM analyses in this time-window was 63 (82%), 63 (82%), and 65 (84%) for NT-proBNP, Troponin T, and CRP, respectively, as a result of the reasons described above. Hazard ratios for developing AR obtained with JM were 0.96 (95% CI 0.55-1.68), 0.67 (0.27-1.69), and 1.44 (0.90-2.96) per ln(unit)), for NT-proBNP, Troponin T, and CRP, respectively.

The results of the analyses that simultaneously incorporated NT-proBNP, Troponin T, and CRP are displayed in Table 3. For weeks 0-12, neither an additive effect nor a multiplicative effect of the 3 biomarkers could be demonstrated. The same was true for weeks 13-52; all hazard ratios were close to unity.

Table 3. Hazard ratios for allograft rejection during weeks 0-12 and weeks 13-52 post-HTx for NT-proBNP, troponin T and CRP combined.

	Hazard ratio (95% confidence interval)	
Variable	**Week 0-12 post-HTx**	**Week 13-52 post-HTx**
NT-proBNP, troponin T and CRP combined by summation	0.95 (0.84-1.07)	0.93 (0.64-1.33)
Age	0.98 (0.95-1.01)	1.01 (0.97-1.06)
Gender	0.66 (0.36-1.21)	1.01 (0.44-2.34)
NT-proBNP, troponin T and CRP combined by multiplication*	0.79 (0.53-1.18)	1.02 (0.97-1.07)
Age	0.98 (0.36-1.20)	1.00 (0.43-2.30)
Gender	0.65 (0.36-1.20)	0.60 (0.15-2.39)

* Ln transformed

DISCUSSION

In this study of 77 HTx recipients, we found that high postoperative NT-proBNP, Troponin T and CRP values decline progressively, reaching a steady state after approximately 12 weeks post-HTx. We did not find any association between the temporal evolutions of the biomarkers, i.e. repeated measurements of these biomarkers, taken before AR was present, and occurrence of clinically significant AR (ISHLT ≥ 2). Such associations were absent both in weeks 0-12 post-HTx and in weeks 13-52 post-HTx.

A substantial decline of NT-proBNP in the initial months after HTx has also been found in previous studies. (9, 15, 16) Some studies have shown that plasma NT-proBNP levels do not normalize even years after HTx. (17, 18) Other studies have found that despite the general decline in NT-proBNP levels after HTx, periodic increase in levels may also be present.(15, 19, 20) It has been suggested that abnormal renal function (15), and high pulmonary capillary wedge pressures (20) may contribute to this increase. On the other hand, a large study of Bader et al.(15) suggested that a role for these clinical variables is less likely in the first 6 months after HTx. A substantial decline post-HTx has also been found for CRP, (9, 21) troponin T, (22-25) and troponin I level(23) in previous studies. In the early post-HTx phase, a temporary elevation of IL-6 and other cytokines is seen, caused by surgical stress, inflammatory stimuli, infection, or myocardial ischemia.(26) As CRP is produced by hepatocytes under the influence of cytokines, including IL-6, it is likely that this affects CRP concentrations post-HTx as well.(21) Concerning troponin T, this elevation might be due to donor heart ischemia(23), perioperative ischemic injury and / or trauma associated with surgical graft transplantation into the recipient.(22)

Several existing studies have measured BNP repeatedly and have examined its association with AR. However, most of these studies have focused on diagnostic and not prognostic value, and have used only the single measurements concomitant to the rejection episodes in their analyses.(15, 27-30) These studies have rendered inconsistent results. Few studies have examined prognostic value of repeated measurements of NT-proBNP or BNP for AR. Arora et al.(9) examined changes in NT-proBNP and did not find any association with acute cellular rejection, while Garrido et al.(8), Kittleson et al.(7), Martinez-Dolz et al.(31) and Damodaran et al. (32) found positive associations between changes in BNP and NT-proBNP and AR. None of these prognostic studies have used all the available biomarker measurements in their analyses. They examined baseline biomarker values or change in biomarkers between two timepoints.

Studies on repeated measurements of troponin T and AR are less abundant, and these studies examined diagnostic, not prognostic, value of troponin T. Some studies (23, 33, 34) were negative, while other were positive.(24, 25, 35) Dengler et al.(25) and White et al.(16) demonstrated that troponin T was elevated in patients with AR occurring more than 3 months postoperatively. Troponin T was only measured in the sample taken at the

moment of biopsy in these studies, leaving the abilities of this marker to predict acute AR unclear. Subsequently, the same group (35) showed that the diagnostic efficiency of troponin T measurement improved markedly when taking into account male recipient gender, recipient age < 60 years, female donor gender and donor age ≥ 33 years. Chance et al. (22) also found that the troponin concentration was significantly higher in patients with rejection than in those without, and concluded that troponin T may have a role to play as an adjunct test to EMB in the diagnosis of AR.

Several studies on the diagnostic value of CRP level for AR have failed to demonstrate significant associations.(22, 36, 37) In contrast, Martinez-Dolz et al.(21) found that CRP showed significant and sustained differences between patients with and without rejection and that low CRP levels ruled out rejection with high specificity. However, they used ROC curves which does not account for correlations between intra-patient measurements. Studies on the prognostic value of CRP include Eisenberg et al, who found that elevated plasma levels of CRP are associated with subsequent allograft failure in cardiac transplant recipients.(38) Only one baseline CRP measurement was performed. Arora et al.(9) examined absolute and percentage change in CRP, and found no association with AR.

In our study, we have included all available serial biomarker measurements up to the first episode of AR in the analysis. Further strengths of the present study include the availability of a large number of biopsies (n=1136) and the consecutive repeated measurement of 3 biomarkers, which represent various pathophysiological pathways underlying AR. Also, we are the first to use JM to examine the prognostic value of repeated biomarker measurements for AR. As described above, previous studies have mostly investigated diagnostic value and have often applied receiver-operating characteristics (ROC) curves, which focus on the biomarker measurement concurrent to the biopsy that shows AR.(21) Studies on prognostic value have generally applied regression models, using the difference between two biomarker measurements as the determinant.(7, 8) While these methods disregard the full temporal evolution of the biomarkers, JM takes into account the total number of repeated measurements available.(11) Since biomarker levels may change at a certain moment in time when a (subclinical) pathological state sets in, this approach results in less bias compared to simplified models. Moreover, JM makes optimal use of the available time-to-event information because it accounts for dependence among repeated observations in an individual patient.(11) ROC curves and simple regression models do not account for such intra-individual correlations and may provide biased results.(39) JM is also appropriate when repeated measurements are performed at varying timepoints and when numbers of available repeated measurements are unequal between patients.(39) A limitation of JM is that it currently only allows for one determinant to be repeatedly measured. Nevertheless, by combining standardized values of NT-proBNP, Troponin T and CRP into one variable, we were able to examine their combined prognostic value. To our best knowledge, this has not been done before.

Some issues relating to this study warrant consideration. Seventy-three percent of our patients experienced at least one rejection episode in the first year post-HTx. Although 1-year rejection rates are known to vary among centers, ranging from 4.5%(40) to 85.6%(41), the rate in our center may be considered as rather high. Such variations in rejection rates are inherent to the fact that the centers performing HTx procedures, as well as the pathology subspecialists who perform the actual scoring of the biopsies, are highly limited in number, and may display center-specific inter-observer variation. Nevertheless, the survival rate post-HTx in our center is known to be very high: 69% at 10 years (unpublished data), compared to an average of 55% at 10 years according to the International Society of Heart and Lung Transplantation(42).

The limitations of the current study include its retrospective nature. Nevertheless, all data required could be collected from the medical records, given that this patient population is under strict clinical surveillance. Furthermore, follow-up was limited to 1 year for the present study. This resulted in a relatively low incidence of mortality (n=5), which could not be used as an endpoint.

In conclusion, high postoperative NT-proBNP, Troponin T and CRP values decline progressively, reaching a steady state after approximately 12 weeks post-HTx. The temporal evolution of these biomarkers does not predict future acute AR both in the early and late course of the first year post-HTx.

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PART V |

Discussion

8

Summary and conclusions

18

Summary and conclusions

Since 'prevention is better than cure', predictive thinking plays an important role in cardiology. After all, appropriate tools for risk stratification in combination with strategies aiming at risk reduction should be available in order to prevent recurrent adverse events and mortality in patients with acquired heart disease. The aim of this thesis was to explore advanced methods for clinical outcome prediction in acquired heart disease, while overarching several methodological, statistical, laboratory and imaging techniques. Specifically, this objective was three fold: To perform a critical appraisal of methodological aspects of existing prediction models used for patients with coronary artery disease (CAD) (Part I); To investigate a wide range of inflammatory biomarkers as potential predictors of adverse events in CAD patients, and to examine their value for assessment of extent and composition of coronary atherosclerosis as assessed by intravascular ultrasound imaging (Part II), and; To evaluate diagnostic and predictive value of several, repeatedly measured, biomarkers in patients with heart failure and heart transplant recipients , while making use of advanced statistical methods (Part III).

PART II: METHODOLOGICAL ASPECTS OF PREDICTION MODELS FOR CLINICAL OUTCOME IN CORONARY ARTERY DISEASE

In Chapter 2.1, we present an overview of existing models for prediction of cardiovascular adverse events in patients with stable, established CAD and discuss (assessment of) model performance. Multiple prediction models have been developed in such patients, however, large variation was present in classic cardiovascular risk factors, other than age and gender, which were included in the different models. Differences in study design, study populations and incidence rates may in part account for this heterogeneity. We found that the majority of existing studies have paid insufficient attention to model performance and validation. Therefore, we set out to develop and validate a series of risk prediction models for different endpoints, both fatal and non-fatal, in a prospective cohort consisting of over 12,000 European patients with established CAD, making use of the EUROPA database (chapter 2.2). To provide an extensive framework for cardiovascular disease prediction, we chose to also examine PCIs and CABGs as outcomes. Most studies that have developed prediction models for CAD patients did not include these outcomes, nor did they examine these outcomes separately. While the performance of our models for the prediction of cardiovascular and non-cardiovascular mortality was comparable to that of previous existing models, we also demonstrated that model performance is considerably worse for non-fatal MI, PCI and CABG examined as separate outcomes.

In **Chapter 3,** we describe an alternative for prognostic modeling that uses regression analyses of isolated patient datasets. Regression models tend to focus on one outcome event, and are usually based on just one baseline evaluation of the patient, thus failing

to take the disease process in its dynamic nature into account. This approach is only suitable to address relatively short-term effects, not long-term predictions. Describing the clinical course of a patient with CAD by a (micro-)simulation model may overcome these limitations. This method simulates the remaining lifetime of one single virtual patient at a time and builds a virtual patient population by repeating the simulation numerous times (Chapter 3.1). Micro-simulation models have already been developed for patients requiring aortic valve replacement (AVR) in order to assist with the choice of an appropriate type of valve, and have been implemented in the software package AVRSim. No such models have yet been developed for patients with established CAD. To investigate whether AVRSim can also be applied to any given type of disease (or disease related event), we developed a new software package, General Microsimulation Toolkit (GMT) (chapter 3.2). This package calculates the time-to-event, event-free period and life expectancy. Moreover, it facilitates the comparison of the outcomes for each for different treatment choices. The GMT model for AVR showed good agreement with the AVRSim package. This implies that GMT may be adapted and applied for prognostic modeling in patients with CAD. Obviously, such new models will need to be evaluated internally and externally before they can be used in daily practice.

PART III: THE ROLE OF INFLAMMATORY BIOMARKERS IN PREDICTION OF CLINICAL OUTCOME IN CORONARY ARTERY DISEASE

In most cases, prediction models for CAD patients are based on established risk factors. However, a considerable part of adverse events during follow-up is not fully explained by these risk factors. Inflammation has been recognized as an important pathophysiological mechanism contributing to CAD. Therefore, insight into the role of inflammatory blood biomarkers in CAD may aid in identifying individuals at high risk of adverse events. In **Part III,** we focus on inflammatory blood biomarkers for the prediction of the extent and composition of coronary atherosclerosis and major adverse cardiac events (MACE). Moreover, we elaborate on the role of smoking, an important inflammatory stimulus.

The studies described were performed within the ATHEROREMO-IVUS study. This study included 581 patients who underwent coronary angiography for acute coronary syndrome (ACS) (n=319) or stable angina pectoris (SAP) (n=263) and in whom intravascular ultrasound (IVUS) imaging of a non-culprit coronary artery was performed. Using virtual histology (VH) IVUS, the extent (plaque volume, plaque burden) and composition (fibrous, fibro-fatty, dense calcium and necrotic core) of coronary atherosclerosis were assessed and high-risk lesions were identified (such as VH-IVUS derived thin-cap fibroatheroma (TCFA), lesions with plaque burden ≥70%, and lesions with minimal luminal area ≤4.0 mm2). Subsequently, these patients were followed-up for MACE for one year.

In chapter 4, we investigated whether several circulating cytokines, chemokines, and acute phase proteins are associated with IVUS-derived measures of plaque burden and features of plaque vulnerability, and whether they are useful for clinical risk stratification with regard to cardiovascular outcome. With regard to cytokines (chapter 4.1), we found that higher circulating tumor necrosis factor alpha (TNF-α) (pro-inflammatory cytokine) and lower circulating interleukin-10 (IL-10) (anti-inflammatory cytokine) are associated with higher coronary plaque burden and with presence of VH-TCFA lesions (for IL-10, in particular 'large' VH-TCFA lesions, i.e. VH-TCFA lesions with a plaque burden ≥ 70%). However, these associations did not translate into a higher incidence of MACE. On the other hand, in has recently been shown in the same study population that presence of lesions with a high plaque burden, and presence of (large) VH-TCFA lesions, are both independently associated with a higher MACE rate. Altogether, these findings imply that the deleterious effects of high TNF-α and low IL-10 do not translate into a higher MACE rate in the current study population. Possible explanations may include the fact that the magnitude of the effect of these biomarkers is small in the context of this multifactorial disease, or that the current study lacks statistical power to expose such an effect.

With regard to chemokines (chapter 4.2) we concluded that higher circulating monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) are associated with higher coronary plaque burden and higher percentages of necrotic core tissue, as well as the presence of TCFA lesions (for MIP-1α, in particular VH-TCFA lesions with a plaque burden ≥ 70%). We did not observe a correlation between MCP-1 and MIP-1α concentration and occurrence of MACE. In patients who were admitted with ACS, lower regulated upon activation normal T cell expressed and secreted (RANTES) concentrations were associated with higher plaque burden. In the overall patient population, RANTES was also found to be independently predictive of the occurrence of adverse outcomes. Its association with acute cardiac events (death or ACS) seemed to be even stronger than with all major adverse cardiac events (death, ACS or unplanned coronary revascularization). This may indicate that RANTES is especially predictive for plaque rupture rather than plaque growth.

In chapter 4.3, we examined acute phase proteins, and demonstrated that higher concentrations of ferritin are associated with higher coronary plaque volume as well as a higher percentage of fibrofatty tissue in the coronary atherosclerotic plaque, the latter only in patients with acute coronary syndrome at baseline. Since serum concentrations of ferritin may vary in a wide range of conditions, the interpretation of ferritin level is complicated. Furthermore, higher concentrations of Alpha-2-Macroglobulin (α2M) were associated with lower percentage of fibrous tissue and higher percentage of dense calcium tissue in the coronary atherosclerotic plaque. However, these associations with plaque composition could not be further translated into associations with number of VH-TCFA

lesions. Finally, Plasminogen Activator Inhibitor 1 (PAI-1) was found to be predictive for the occurrence of MACE, which is in line with prior studies.
Altogether, these in-vivo findings suggest that the above-mentioned inflammatory biomarkers may play a role in extent, advanced phenotype and vulnerability of coronary atherosclerosis. With regard to risk stratification on cardiovascular outcome, lower circulating RANTES and higher circulating PAI-1 were the only biomarkers from the panel that we investigated, which displayed potential predictive value.
In Chapter 5.1, we focused on cigarette smoking and its association with the extent and vulnerability of coronary atherosclerosis. Smoking showed a tendency towards slightly higher coronary plaque burden. However, no associations could be demonstrated with percentage necrotic core, nor with VH-IVUS derived TCFA lesions, suggesting that smoking has no major influence on plaque vulnerability. Lack of such an association may in part be explained by the possibility that plaque erosion, and not as much vulnerable plaque rupture, is the intermediate between smoking and cardiac adverse events, as suggested by earlier, histopathological studies.

PART IV: THE ROLE OF BIOMARKERS IN PREDICTION OF CLINICAL OUTCOME IN HEART FAILURE AND AFTER HEART TRANSPLANTATION

CAD remains by far the most common cause of heart failure. Due to improved treatment, CAD mortality rate has declined, but survivors are often left with damaged myocardium resulting in left ventricular dysfunction and, potentially, clinical heart failure. Blood biomarkers may potentially be a valuable tool in risk stratification of these patients.
Although many studies have investigated the diagnostic and predictive value of new biomarkers in heart failure, the majority of these studies included patients with heart failure with reduced ejection fraction (HFREF) only. Limited data are available on biomarkers in heart failure with normal ejection fraction (HFNEF), in spite of its ominous prognosis. In Chapter 6.1, we provide an overview of blood biomarkers that have been found to be associated with the occurrence and prognosis of HFNEF. Several biomarkers and biomarker categories, including biomarkers of myocyte stress, inflammation, extracellular matrix remodeling, growth differentiation factor 15 (GDF-15), cystatin C, resistin, and galectin-3, appear to be potentially promising diagnostic tools in HFNEF. Some of them, including TNF-a, interleukin-6, amino-terminal propeptide of procollagen type I (PINP), amino-terminal propeptide of procollagen type III (PIIINP), osteopontin, and cystatin C, may carry predictive value as well.
In patients with end stage heart failure, treatment options have improved in recent years and include a combination of drugs, mechanical devices and surgical procedures which may improve symptoms and survival. Nevertheless, for some patients these treatment options are not sufficient, and these patients might benefit most from heart transplantation.

Acute allograft rejection (AR) is a common problem after heart transplantation (HTx) and is one of the major causes of cardiac graft loss. In **Chapter 7**, we examine diagnostic and predictive value of the biomarkers NT-pro-B-type natriuretic peptide (NT-proBNP), cardiac troponin T and C-reactive protein (CRP) for AR. Existing studies on this topic have provided inconsistent results, in part due to differences in methodology. Of further note is that the methodology applied by these studies was not always appropriate.
We found that CRP level carries diagnostic value for concomitant presence of AR in the first year after HTx (chapter 7.1). However, discriminative ability of CRP was too poor to replace endomyocardial biopsy. To assess the prognostic value (chapter 7.2), we used so-called Joint Modeling (JM), with combines mixed models (in our case for serial biomarker measurements) with Cox proportional hazards models (in our case for the risk of AR). Joint modeling makes optimal use of the available time-to-event information because it accounts for dependence among repeated observations in an individual patient. We found that none of the three biomarkers we investigated were able to predict future AR during the first year after HTx.

CONCLUSIONS

The main conclusions of this thesis may be summarized as follows:

- Existing prediction models for adverse cardiac events in patients with CAD have mostly used regression analyses and have paid limited attention to model performance and validation. Micro-simulation is a promising method that could improve prediction models by taking into account the dynamic nature of the disease process.
- Several cytokines (TNF α, IL-10), chemokines (MCP-1, MIP-1α, RANTES) and acute phase proteins (ferritin, α2M) are associated with various aspects of extent and composition of coronary atherosclerosis, and RANTES and PAI-1 are associated with occurrence of MACE. Thus, these inflammatory biomarkers are potential candidates whose incremental value should be evaluated for prediction models of recurrent events in CAD patients
- Several biomarkers and biomarker categories, including biomarkers of myocyte stress, inflammation, extracellular matrix remodelling, GDF-15, cystatin C, resistin, and galectin-3, appear to be potentially promising diagnostic tools in HFNEF. Some of them, including TNF-a, IL-6, PINP, PIIINP, osteopontin, and cystatin C, may carry predictive value as well.
- CRP level carries diagnostic value for concomitant presence of AR in the first year after heart transplantation. However, discriminative ability of CRP is too poor to replace endomyocardial biopsy. None of the repeatedly measured biomarkers which were investigated (NT-proBNP, troponin T nor CRP) was able to predict future AR during the first year after heart transplantation.

FUTURE DIRECTIONS

The above findings illustrate that combining advanced laboratory, imaging and statistical techniques may lead to additional insights into prediction of adverse cardiovascular events in patients with acquired heart disease. As such, further research combining these aspects is warranted. An example of an ongoing study that covers a wide range of sophisticated, multidisciplinary methods is the 'Serial Biomarker meaSurements and new echocardiographic techniques in chronic Heart Failure patients result in Tailored prediction of prognosis' (Bio-SHiFT) study. In this study, patients with chronic heart failure are included, blood samples are drawn every 3 months during a follow-up period of up to 2,5 years and stored for future biomarker determination, and echocardiography (including TDI, Speckle tracking and 3D-echocardiography) is performed repeatedly. The results of this study are expected to provide information on individual patterns of change in biomarker levels and cardiac structure and function, and may thus carry potential to improve individual risk prediction and to herewith contribute to personalized medicine.

SAMENVATTING EN CONCLUSIES

Omdat voorkomen gewoonlijk beter is dan genezen, speelt voorspellend denken een belangrijke rol in de cardiologie. Veelal worden daarbij schema's of 'modellen' gebruikt die kenmerken van patiënten met hart- en vaatziekten koppelen aan het risico op medische (nood)situaties, zoals hartinfarct, herseninfarct of overlijden (engelse afkorting: *MACE*). De behandeling wordt vervolgens afgestemd op de individuele situatie van de patiënt, rekening houdend met de hoogte van genoemd risico.

Het is evident dat de kwaliteit van de risico-voorspelling en daarmee (indirect) die van de behandeling afhankelijk is van de prestatie van het gebruikte model. Dit proefschrift had zowel ten doel de prestaties in kaart te brengen van bestaande risico-voorspellingsmodellen voor patiënten met verworven hart- en vaatziekten, als ook om deze, waar mogelijk, te verbeteren. Daarbij is gebruik gemaakt van geavanceerde statistische methoden, intravasculaire beeldvorming en laboratorium technieken voor het bepalen van zogenaamde bloed *biomarkers*.

Deel I van dit proefschrift bevat een kritische beoordeling van methodologische aspecten van bestaande risico-voorspellingsmodellen die gebruikt worden voor patiënten met coronaire hartziekte (CAD). In deel II wordt een breed scala aan inflammatoire *biomarkers* onderzocht als mogelijke voorspellers van het beloop van coronaire atherosclerose, zowel als van het ontstaan van medische (nood)situaties bij CAD patiënten. Deel III beschrijft de diagnostische en voorspellende waarde van verschillende, herhaaldelijk gemeten, *biomarkers* bij patiënten met hartfalen en bij patiënten die een harttransplantatie hebben ondergaan.

DEEL II: METHODOLOGISCHE ASPECTEN VAN VOORSPELLENDE MODELLEN VAN KLINISCHE UITKOMST BIJ CORONAIRE HARTZIEKTE

In hoofdstuk 2.1 presenteren we een overzicht van de bestaande risico-voorspellingsmodellen voor cardiovasculaire (nood)situaties bij stabiele CAD patiënten. Tevens bespreken en beoordelen we de model prestaties. Voor deze patiëntengroep zijn er meerdere risico-voorspellingsmodellen ontwikkeld. Leeftijd en geslacht worden in alle modellen als risicofactor gebruikt, maar er bestaat grote variatie in het gebruik van de overige klassieke cardiovasculaire risicofactoren. Deze heterogeniteit kan deels verklaard worden door verschillen in onderzoeksopzet, onderzoekspopulatie en incidentie. We vonden dat het merendeel van de bestaande studies onvoldoende aandacht hebben besteed aan model prestatie en validatie. Om deze reden hebben we besloten een reeks risico-voorspellingsmodellen te ontwikkelen en te valideren voor verschillende, zowel fatale als niet-fatale, eindpunten. Om deze modellen te ontwikkelen hebben we een

prospectief cohort gebruikt, bestaande uit meer dan 12.000 Europese CAD patiënten, geselecteerd uit de EUROPA-database (hoofdstuk 2.2).
Om de risico-voorspellingsmodellen voor hart- en vaatziekten zo optimaal mogelijk te kunnen onderzoeken hebben we ervoor gekozen om ook de PCI's en CABG's als uitkomsten mee te nemen. De meeste studies die risico-voorspellingsmodellen hebben ontwikkeld voor CAD patiënten hebben deze uitkomsten niet onderzocht als gecombineerd eindpunt, noch afzonderlijk van elkaar. We hebben aangetoond dat de prestatie van onze modellen voor het voorspellen van cardiovasculaire en niet-cardiovasculaire doodsoorzaken vergelijkbaar zijn met die van eerdere bestaande modellen. Daarnaast hebben we laten zien dat de model prestaties voor de niet-fatale uitkomsten MI, PCI en CABG als afzonderlijke uitkomsten, aanzienlijk slechter zijn.
In hoofdstuk 3 beschrijven we zogenaamd micro-simulatie als een alternatieve benadering voor risico-schatting bij CAD patiënten. Regressiemodellen hebben de neiging zich te richten op slechts één uitkomstmaat, terwijl zij meestal gebaseerd zijn op slechts één evaluatiemoment van de patiënt, namelijk aan het begin van de studie. Hierdoor slagen deze modellen er niet in om rekening te houden met het veranderingen die tijdens het ziekteproces kunnen optreden. Bijvoorbeeld, een patiënt die aan het begin van de studie geen diabetes had kan dit tijdens de studie wel ontwikkelen. Dit zorgt ervoor dat deze benadering alleen geschikt is voor het voorspellen van relatief korte termijn effecten, en niet voor lange termijn voorspellingen. Een (micro-) simulatiemodel, die het klinisch verloop van een patiënt met CAD beschrijft kan dan uitkomst bieden. Dit model is namelijk in staat om het klinisch verloop te simuleren. Van een individuele patiënt kan de resterende levensduur worden gesimuleerd. Door deze simulatie vele malen te herhalen kan een virtuele patiëntenpopulatie worden gecreëerd van (bij aanvang) identieke patiënten (hoofdstuk 3.1). Op deze wijze kan het klinisch verloop van een individuele patiënt worden ingeschat, waarbij rekening wordt gehouden met medische situaties die zich 'onderweg' voordoen. Voor patiënten die aortaklepvervanging (AVR) moeten ondergaan waren al micro-simulatiemodellen ontwikkeld. Deze modellen helpen met de keuze van de meest geschikte type klep en zijn in het softwarepakket AVRSim geïmplementeerd en gevalideerd. Voor het bouwen van micro-simulatie modellen bij CAD patiënten ontwikkelden we een nieuw, generiek softwarepakket, "*General Microsimulatie Toolkit*" (GMT) (hoofdstuk 3.2). Dit pakket is in staat om de *time-to-event*, *event-free period* en de levensverwachting te berekenen. Verder kan het op een eenvoudige wijze een vergelijking maken van de resultaten voor verschillende behandelkeuzes. Wij hebben het AVR probleem uitgewerkt met het GMT pakket en het vergeleken met AVRSim. Beide resultaten kwamen goed overeen. . Bij vervolgonderzoek kan GMT gebruikt worden voor risico-voorspelling bij patiënten met CAD.

DEEL III: DE ROL VAN INFLAMMATOIRE BIOMARKERS BIJ HET VOORSPELLEN VAN DE KLINISCHE UITKOMST VAN CORONAIRE HARTZIEKTE

In de meeste gevallen worden risico-voorspellingsmodellen voor het optreden van *MACE* bij CAD patiënten ontwikkeld op basis van bekende risicofactoren, zoals bijvoorbeeld roken, suikerziekte en hoge bloeddruk. Veel *MACEs* die zich tijdens de follow-up voordoen kunnen echter niet voorspeld worden op basis van bekende risicofactoren. Er is dus ruimte voor verbetering van risico-voorspellingsmodellen. Wij hebben die gezocht bij verschillende pathofysiologische concepten. Inflammatie is een erkend pathofysiologisch mechanisme dat een bijdrage levert aan het ontstaan van CAD. Daarom kan het verkrijgen van inzicht in de rol van inflammatoire *biomarkers* helpen bij het identificeren van mensen met CAD die een hoog risico hebben op het optreden van *MACEs*. In deel III richten we ons op inflammatoire *biomarkers* bij het voorspellen van het optreden en de ernst van een *MACE*. Daarnaast onderzoeken we een belangrijke stimulus die aanzet tot een inflammatoire respons, namelijk roken.

Voor de onderzoeken uit deel III hebben we gebruik gemaakt van de data uit de ATHEROREMO-IVUS studie. Deze studie omvatte 581 patiënten die coronaire beeldvorming ondergingen middels angiografie vanwege een *"acuut coronair syndroom"* (ACS) (n = 319) of *"stabiele angina pectoris"* (SAP) (n = 263) en bij wie intravasculaire beeldvorming (IVUS) was uitgevoerd van een kransslagader. Met behulp van virtuele histologie (VH) IVUS, werd de mate (*plaque volume, plaque burden*) en samenstelling (*fibrous, fibro-fatty, dense calcium and necrotic core*) van coronaire atherosclerose beoordeeld en werden hoog-risico laesies geïdentificeerd (zoals *"thin-cap fibroatheroma"* (TCFA) laesies, *plaque burden* van ≥ 70%, en letsels met minimale luminale oppervlakte ≤ 4,0 mm^2). Vervolgens werd bij deze patiënten gekeken of er een *MACE* optrad in het eerste jaar na de ingreep. In hoofdstuk 4 hebben we onderzocht of een aantal circulerende *biomarkers*, namelijk cytokines, chemokines en acute fase eiwitten, kunnen worden geassocieerd met metingen verricht met IVUS. Voor cytokinen (hoofdstuk 4.1), vonden we dat hogere waardes van circulerend tumor necrose factor alfa (TNF-α) (een pro-inflammatoire cytokine) en lagere waardes van circulerend interleukine-10 (IL-10) (een anti-inflammatoire cytokine) geassocieerd waren met hogere coronaire *plaque burden* en de aanwezigheid van VH-TCFA laesies. Bij IL-10 betrof het voornamelijk "grote" VH-TCFA laesies, namelijk VH-TCFA leasies met een *plaque burden* van ≥ 70%. Echter, de hoge waardes van TNF-α en lage waardes IL-10 lieten zich echter niet vertalen in hogere percentages *MACE* in de huidige studiegroep. Mogelijke verklaringen kunnen zijn dat de grootte van het effect van deze *biomarkers* slechts klein is in de context van deze multifactoriële ziekte ofwel dat de huidige studie statistische power mist om een dergelijk effect aan te tonen.

Wat de door ons onderzochte chemokinen betreft (hoofdstuk 4.2), vonden wij dat hogere waardes van circulerend monocyt chemoattractant eiwit-1 (MCP-1) en macrofaag inflammatoir proteïne-1α (MIP-1α) geassocieerd zijn met hogere coronaire *plaque burden*, hogere percentages *necrotic core*, en de aanwezigheid van TCFA laesies (voor MIP-1α betreft het vooral VH-TCFA letsels met een *plaque burden* van ≥ 70%). We hebben geen associatie gevonden tussen de concentratie van MCP-1 en MIP-1α en het optreden van *MACE*.

Bij patiënten die werden opgenomen met ACS, waren lagere concentraties van regulated upon activation normal T cell expressed and secretes (RANTES) geassocieerd met de aanwezigheid van hogere *plaque burden*. ACS en niet-ACS patiënten samengenomen, bleek de RANTES concentratie ook een onafhankelijke voorspeller voor het optreden van *MACE*. De associatie met acute cardiale gebeurtenissen (overlijden of ACS) leek nog sterker te zijn dan bij alle andere belangrijke cardiovasculaire gebeurtenissen (overlijden, ACS of ongeplande coronaire revascularisatie). Dit kan erop wijzen dat de RANTES concentratie vooral voorspellend is voor een plaque ruptuur in plaats van plaque groei.

In hoofdstuk 4.3 onderzochten we de acute fase eiwitten. Hogere concentraties van ferritine waren geassocieerd met een hoger coronair *plaque volume* alsmede, in ACS patiënten, een hoger percentage van *fibrofatty* weefsel in de coronaire atherosclerotische plaque Aangezien de concentraties van ferritine in het serum verhoogd kunnen zijn bij verschillende medische omstandigheden, bijvoorbeeld bij een acute infectie of bij patiënten met chronische nierfalen, is de interpretatie van een verhoogde ferritine concentratie niet triviaal. Een hogere concentratie van alfa-2-Macroglobuline (a2M) was geassocieerd met een lager percentage bindweefsel en een hoger percentage *calcium tissue* in de coronaire atherosclerotische plaque.

Samenvattend, deze resultaten suggereren dat de hierboven genoemde inflammatie *biomarkers* een rol spelen in de fenotypering (verzameling van waarneembare kenmerken) en kwetsbaarheid van coronaire atherosclerose. Met betrekking tot de risico inschatting van *MACE*, waren lagere waardes van het circulerend RANTES en hogere waardes van circulerend PAI-1 de enige onderzochte *biomarkers* die potentiële voorspellende waarde hadden.

In hoofdstuk 5.1, hebben we ons gericht op de relatie tussen het rookgedrag van patiënten en de met behulp van VH-IVUS in beeld gebrachte coronaire atherosclerose. Rokers lieten een trend zien naar hogere coronaire *plaque burden*. Er kon echter geen associatie worden aangetoond met het percentage van *nectrotic core*, noch met VH-IVUS gemeten TCFA laesies, wat suggereert dat roken geen grote invloed heeft op de kwetsbaarheid van de plaque. Het ontbreken van een dergelijk verband kan deels worden verklaard doordat de plaque mogelijk eerder wordt aangetast, maar niet zozeer scheurt.

DEEL IV: DE ROL VAN BIOMARKERS IN HET VOORSPELLEN VAN DE KLINISCHE UITKOMST BIJ HARTFALEN EN NA HARTTRANSPLANTATIE

Het acute hartinfarct de meest voorkomende oorzaak van hartfalen. Als gevolg van betere behandelingen tijdens de acute fase, is het sterftecijfer na een hartinfarct gedaald. Helaas hebben mensen die overleven vaak een beschadigde hartspier. Dit leidt tot een verslechterde linkerkamerfunctie en klinisch hartfalen. Bloed *biomarkers* kunnen mogelijk een waardevol instrument zijn bij de risico inschatting van deze patiënten.

De meeste studies die de diagnostische en voorspellende waarde van *biomarkers* bij hartfalen hebben onderzocht, richtten zich op patiënten met een verminderde ejectiefractie (HFREF). Er zijn slechts beperkte gegevens beschikbaar over *biomarkers* in patiënten met hartfalen met normale ejectiefractie (HFNEF). In hoofdstuk 6.1, geven we een overzicht van de bloed *biomarkers* die worden geassocieerd met het optreden en de prognose van HFNEF. Verscheidene *biomarkers* en *biomarker* categorieën, waaronder *biomarkers* voor myocyten stress, ontstekingen, extracellulaire matrix remodelering, groei differentiatiefactor 15 (GDF-15), cystatine C, resistin en galectine-3, lijken veelbelovend voor diagnostische toepassingen. De *biomarkers* TNF-a, interleukine-6, "amino-terminale propeptide van het type I procollageen" (PINP), "amino-terminale propeptide van type III procollageen" (PIIINP), osteopontine en cystatine C kunnen ook prognostisch van belang zijn. De afgelopen jaren zijn de behandelopties voor patiënten met eindstadium hartfalen verbeterd. Symptomen kunnen worden verlicht, en overleving verbeterd door een combinatie van medicijnen, mechanische ondersteuning van het hart en chirurgische behandelingsopties. Helaas zijn deze behandelingen niet voor alle patiënten toereikend. Deze 'uitbehandelde' patiënten zouden profijt kunnen hebben van een harttransplantatie (HTx). Acute allograft afstoting (AR) is een veel voorkomend probleem na HTx en is een van de belangrijkste oorzaken van verlies van het harttransplantaat. In hoofdstuk 7 onderzoeken we diagnostische en voorspellende waarde van de *biomarkers* NT-pro-B-type natriuretisch peptide (NT-proBNP), troponine T en C-reactief proteïne (CRP) voor AR. Bestaande studies over dit onderwerp bevatten inconsistente resultaten, mede als gevolg van verschillen in methodologie. Daarnaast was bij deze studies niet altijd duidelijk welke methodologie was toegepast.

Wij vonden dat het optreden van AR in het eerste jaar na HTx samenging met een hogere concentratie van CRP(hoofdstuk 7.1). Echter, het diagnostisch vermogen van CRP was te gering om endomyocardiale biopsie te vervangen. Om de prognostische waarde (hoofdstuk 7.2) te beoordelen, gebruikten we een geavanceerde statistische techniek die *Joint Modeling* (JM) wordt genoemd. Deze methode houdt rekening met herhaalde metingen in een individuele patiënt, maar ook met verschillen tussen de patiënten in de database. We vonden dat geen van de onderzochte *biomarkers* gebruikt konden worden voor het voorspellen van AR gedurende het eerste jaar na HTx.

CONCLUSIES

De belangrijkste conclusies van dit proefschrift zijn de volgende:

- Bestaande risico-voorspellingsmodellen voor ongunstige cardiale uitkomsten bij patiënten met CAD gebruiken veelal regressie-analyses en besteden slechts beperkte aandacht aan de prestatie en validatie van de modellen. Micro-simulatie houdt rekening met de dynamische aard van het ziekteproces en is daarom een veelbelovende methode die risico-voorspellingsmodellen zou kunnen verbeteren.
  - Verschillende cytokinen (TNF α, IL-10), chemokinen (MCP-1, MIP-1α, RANTES) en acute fase eiwitten (ferritine, a2M) zijn geassocieerd met verschillende aspecten van de omvang en samenstelling van coronaire atherosclerose (gemeten met VH-IVUS), terwijl RANTES en PAI -1 geassocieerd zijn met het optreden van *MACE*. Deze inflammatoire *biomarkers* zijn daarom potentiële kandidaten om te worden opgenomen in voorspellingsmodellen voor het optreden van *MACE* bij CAD patiënten. Verder onderzoek in dit verband is nuttig en nodig.
- Verschillende *biomarkers* en *biomarker* categorieën, waaronder *biomarkers* voor myocyte stress, inflammatie, extracellulaire matrix remodelering, GDF-15, cystatine C, resistin en galectin-3, lijken veelbelovende diagnostische hulpmiddelen in HFNEF. Sommige, waaronder TNF-a, IL-6, PINP, PIIINP, osteopontine en cystatine C kunnen ook als hulpmiddel dienen bij het voorspellen van prognose.
- Het optreden van van AR in het eerste jaar na harttransplantatie gaat samen met een verhoogde CRP concentratie. Echter, het diagnostisch vermogen van CRP is te gering om endomyocardiale biopsie te vervangen. Geen van de herhaaldelijk gemeten *biomarkers* die werden onderzocht (NT-proBNP, troponine T of CRP) kon voorspellen of er gedurende het eerste jaar na harttransplantatie AR zou optreden.

TOEKOMSTIGE RICHTINGEN

De bovenstaande bevindingen illustreren dat het combineren van geavanceerde laboratorium technieken, beeldvorming en statistische technieken kunnen leiden tot aanvullende inzichten in de voorspelling van een medische (nood)situatie bij patiënten met verworven hart- en vaatziekten. Daarom is verder onderzoek, die deze aspecten combineert, wenselijk. Een voorbeeld van een lopend onderzoek dat een breed scala van geavanceerde, multidisciplinaire methoden gebruikt, is de Serial Biomarker meaSurements and new echocardiographic techniques in chronic Heart Failure patients result in Tailored prediction of prognosis' (Bio-shift) studie. In deze studie worden patiënten met chronisch hartfalen geïncludeerd. Bloedmonsters worden tijdens een follow-up periode van maximaal 2,5 jaar elke 3 maanden verzameld en opgeslagen voor toekomstig *biomarker* onderzoek, tevens wordt er herhaaldelijk echografisch onderzoek uitgevoerd. De verwachting is dat de

resultaten van deze studie informatie zullen geven over individuele veranderingspatronen betreft *biomarkers* en de structuur en functie van het hart, en hiermee potentie heeft om de individuele risicovoorspelling te verbeteren en bijdraagt aan geneeskunde die zich specifiek richt op de patiënt in kwestie.

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CURRICULUM VITAE

Linda Charlotte Battes werd op 26 maart 1984 geboren te Leidschendam. In 2001 slaagde zij voor het eindexamen HAVO aan het Alfrink College te Zoetermeer. En in 2003 slaagde zij voor het eindexamen VWO aan het Alfrink College te Zoetermeer. Zij studeerde geneeskunde aan de Erasmus Universiteit Rotterdam en behaalde het doctoraal examen in 2008. Haar keuze co-schap op de intensive care volgde zij in het Amphia ziekenhuis te Breda. Aansluitend volgde zij het oudste co-schap cardiologie eveneens in het Amphia ziekenhuis te Breda. In oktober 2010 behaalde zij het artsexamen en startte haar promotieonderzoek dat heeft geleid tot dit proefschrift in het Erasmus MC te Rotterdam (Prof.dr. H. Boersma). Op 1 april 2014 begon zij als ANIOS cardiologie in het Erasmus MC te Rotterdam.
Linda Charlotte Battes heeft een zoon, Sander (1 jaar).