Repeated Biomarker Measurements in Acquired Heart Disease

Values and Limitations

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Repeated Biomarker Measurements in Acquired Heart Disease
Values and Limitations

Herhaalde biomarkermetingen bij verworven hartaandoeningen
Bruikbaarheid en beperkingen

Proefschrift

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Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. A.L. Bredenoord

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Chapter 1

General introduction

Introduction

Cardiovascular disease, including coronary artery disease (CAD) and heart failure (HF), is the leading cause of death globally[1] and accounts for approximately 1 in every 3 deaths in the western world[2]. In the Netherlands, CAD and HF accounted for 5.4% and 4.8% of all the deceased in 2019[3].

CAD can manifest diversely, from stable angina to nonfatal acute coronary syndrome (ACS), or even as sudden cardiac arrest. The disease is caused by atherosclerosis, a chronic progressive process in which lipid deposits form in the intimal layer of the coronary arteries, under the influence of chronic inflammation. These atherosclerotic plaques cause narrowing of the coronary arteries, which in turn may lead to ischemia of the myocardium and clinical symptoms. If a plaque ruptures, this results in a local thrombotic reaction, which can cause an acute blockage of blood flow, clinically presenting as an acute ACS.

Heart failure (HF) is a clinical syndrome characterized by typical symptoms such as breathlessness, ankle swelling and fatigue. The main causes of HF are CAD, hypertension and obesity[4]. These diseases may induce structural and/or functional cardiac abnormality, resulting in a reduced cardiac output during stress or even at rest[4]. Cardiac output is defined as the product of stroke volume and heart rate, and the human body can activate several mechanisms to optimize these components. Although this process is beneficial during the initial phase of HF and in other situations where the body is in need of more blood flow (e.g. intensive physical exercise or hemorrhage), the long-term effects serve to worsen HF in a vicious cycle[5].

Despite major improvements in treatment, both morbidity and mortality remain high in patients after a first coronary event such as ACS or after an early-stage HF diagnosis. According to the WHO, survivors of a myocardial infarction (MI) have a 5% annual death rate. This is a six-fold increase compared to persons of the same age without CAD[6]. The mortality of HF patients is even higher, with approximately 50% of the patients dying within 5 years after diagnosis[2]. Moreover, after each hospital admission for HF, the chance of new hospital admission and cardiovascular death rises dramatically[7,8].

Against this background, over the past few decades researchers have been trying to better characterize patients with CAD or HF with the highest risk of recurrent events or mortality. Adequate risk estimations could help patients, their families and clinicians decide on the appropriate type and timing of therapies. Moreover, this may improve the cost-efficiency of health care. In addition, epidemiological research on risk predictors for CAD and HF patients, may offer new insights into the pathophysiology of their acquired heart disease[9]. One of the most successful and most used series of risk prediction models are the GRACE-risk models. These models contain several clinical characteristics and are used to predict future risk of adverse events after an initial MI. More specifically, they can be used to predict both the risk of in-hospital and six-month mortality, as well as the risk of the composite of mortality and non-fatal MI within six months[10-12]. The GRACE models have been validated thoroughly[13] and using them for risk prediction after MI is recommended by the guidelines from the ESC[14,15]. When examining the accuracy of the different GRACE-risk models more closely, there is a striking difference in performance of the models that predict mortality alone and those that predict the combined endpoint of recurrent MI and mortality. The C-statistic, a common measure for comparing model accuracy, for in-hospital and six-month death are 0.83[12] and 0.81[10] respectively, while the C-statistic for the combined endpoint within 6 months is only 0.70[11]. The same phenomenon is seen for prediction models of adverse events in patients after HF diagnosis. A meta-analysis of 117 prognostic models revealed on average an overall moderate accuracy for mortality (C-statistic of 0.71), whereas models that predicted a combined endpoint of death or hospitalization had on average a much poorer discriminative ability (C-statistic of 0.63)[16].

Risk-prediction models often consist of patient characteristics combined with one or more so-called 'biomarker' measurements to predict the chance of morbidity and/or mortality in a certain time frame. Although the FDA defined a biomarker as 'a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions'[17], in this thesis the term biomarker usually refers to measurements of molecules or proteins in blood samples, performed by clinical chemistry laboratories. It is not hard to imagine that a single biomarker measurement cannot capture individual differences in the course of complex heterogeneous acquired heart diseases such as CAD and HF. One solution to this

problem used in risk prediction, is using measurements from multiple blood biomarkers, reflecting different aspects of the pathophysiology and/or health status of the patients. Although this approach has indeed led to an increased precision of prediction models[18-22], current risk prediction models still have important limitations. Patient characteristics and biomarker levels are typically only measured once in time, either during the acute phase of the disease, for example at admission for ACS, or after the disease has stabilized. Particularly when time passes since the initial measurement, accuracy of any predictor will most likely decrease as the measurement no longer reflects the current health status of the patients. Moreover, research has shown that if a biomarker concentration in a blood sample is measured twice or the biomarker concentrations is measured twice within the same (stable) patient with blood samples taken a certain period apart, the results will show variability as biomarkers always vary around a patients habitual value. Since in risk prediction models biomarker are typically measured once, this variability around the habitual value will lead to misclassification of the patients' risk for future events (Figure 1.1).

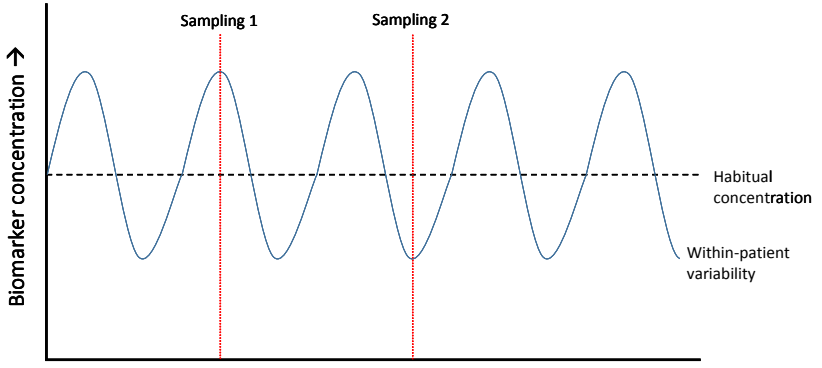

Figure 1.1 Concentrations of a hypothetical biomarker within one stable patient over time. Although the habitual value remains constant over time, a large difference in measured concentrations can be found depending on the timing of the blood sampling. Sampling 1 and sampling 2 characterize the two extreme concentrations that can be measured within this patient.

Because of these important limitations of current biomarker research for prognostication in patients with acquired heart disease, which mostly focuses on one-time measurements, our research group has focused on longitudinal follow-up

with multiple blood samples and echocardiograms taken over time. This allows us to investigate differences in biomarker patterns between patients with recurrent events, such as second ACS or death, and patients without events.

In addition, because of the repeated measurements, we can adjust for the variability of biomarker measurements around the habitual biomarker concentration of a patient. Using this information, we can potentially identify not only vulnerable patients but also vulnerable periods for adverse events within patients during follow-up. For example, in the Bio-SHiFT (Serial Biomarker meaSurements and new echocardiographic techniques in chronic HF patients result in Tailored prediction of prognosis) study, our group previously demonstrated that N-terminal -pro hormone brain natriuretic peptide (NT-proBNP) blood concentrations were systematically elevated in patients with recurrent events compared to event-free patients (vulnerable patient), but also that the concentrations further increased in the period prior to hospitalization (vulnerable period) (Figure 1.2)[23]. In this thesis, I aimed to further explore the potential of using repeated measurements for improving risk prediction, both in patients with ACS and in patients with chronic HF.

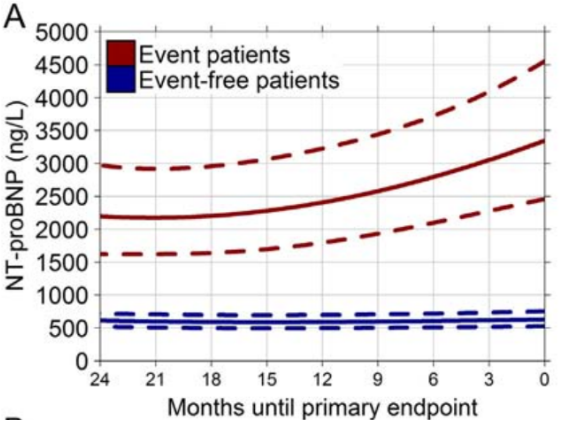

Figure 1.2 Average temporal pattern of NT-proBNP in the Bio-SHiFT study, displayed as time until event. Adjusted figure from "Toward personalized risk assessment in patients with chronic heart failure: Detailed temporal patterns of NT-proBNP, troponin T, and CRP in the Bio-SHiFT study" by Nick van Boven et al. Used with permission.

Aims of this thesis

- To investigate the variability of several key blood biomarkers that have been proposed to serve as predictors used to identify patients at high-risk for recurrent events after an MI.
- To investigate the incremental predictive value of repeated blood biomarker measurements compared to a one-time measurement in patients with an ACS.
- To investigate if repeating echocardiograms and blood sampling in patients with chronic HF leads to better prediction of hospitalization or cardiac death compared to a one-time measurement.

For this purpose, in this thesis, we examined in detail biomarker patterns in patients with CAD and HF. In **part I**, we aimed to evaluate the variability of several key blood biomarkers in cardiology when repeatedly assessed in clinically stable post-ACS patients. Biomarker levels are not only influenced by a patient's medical condition but also by biological variability of biomarkers within a patient and the analytical imprecision of the test used. This variability is clinically relevant, as it can induce changes in treatments of patients and compromise risk stratification in prognostic models, especially when only a single measurement is used. In **part II**, we examine the longitudinal patterns of several known and more experimental biomarkers and their association with the combined endpoint of re-ACS and cardiac death during one year of follow-up in patients with an ACS. Finally, in **part III**, we examine the added prognostic value of repeated echocardiograms compared to a single echocardiograms in patients with chronic HF. We also study the association between these repeated echocardiograms and simultaneously measured blood biomarkers. In addition, using blood samples from the same study, we measure the concentrations of 4 biomarkers reflecting the process of fibrinolysis and 12 adhesion molecules over time using a multiplex biomarker panel. These longitudinal biomarker patterns are then associated with the clinical outcomes in these patients with chronic HF.

In order to perform this research, we have used data from the BIOMArCS (BIOMarker study to identify the Acute risk of a Coronary Syndrome) study and the Bio-SHiFT study.

BIOMArCS

The BIOMArCS study is a prospective, multicenter observational study, in patients admitted with ACS, who were followed-up for one year with high-frequency blood sampling[24]. The BIOMArCS study has been designed to investigate biomarker patterns in detail and their association with clinical outcome in the first year after hospital admission for ACS. Included patients were all admitted to the hospital with an ACS, aged above 40 years, and had at least one cardiovascular risk factor. Patients with estimated glomerular filtration rate <30 ml/min/1.73 m^2 were excluded. Preferably, patients were enrolled during hospital admission, but inclusion at the first outpatient visit post-discharge (usually 4-6 weeks later) was allowed. Venipuncture was performed at admission, discharge, and subsequently every 2 weeks during the first half-year and monthly thereafter. In a small subset of 68 patients, we performed additional blood sampling on day 1 to 4 with the aim to study post-ACS kinetics. The study endpoint was a combination of cardiac death and recurrent ACS.

In total, 844 patients were enrolled in BIOMArCS of whom 45 reached the study endpoint. For cost-efficacy reasons, we limited the number of biomarker tests in endpoint-free patients, while maintaining all available information in study endpoint cases. In order to do so, a case-cohort analysis was used. A random sample of 150 patients was selected from the full dataset of 844 patients, which included 8 patients who reached the study endpoint. Hereafter, the random sample was enriched with the remaining 37 endpoint cases, so that the case-cohort analysis set consists of (all) 45 endpoint cases and 142 endpoint-free patients. Finally, our analysis set was enriched with the blood samples from the 68 patients that underwent additional samples during the first 4 days. As 19 were already included in the (45+142) 187 patients, blood samples from a total of 236 BIOMArCS patients were available. They contributed a median of 7 (25^{th}-75^{th} percentile 5-10) repeated samples per patient, totalling 1775 samples (Figure 1.3) [24,25].

Bio-SHiFT

The Bio-SHiFT study is a prospective, observational study conducted in the Erasmus MC, Rotterdam, the Netherlands, and the Noordwest Ziekenhuisgroep, Alkmaar, the Netherlands. The study enrolled a total of 398 stable outpatients with chronic HF. All patients were aged above 18 and were diagnosed >3 months ago according

to the definition of HF used by the European Society of Cardiology. Both HF patients with a reduced ejection fraction and with preserved ejection fraction were allowed. Exclusion criteria were: need for dialysis, known moderate or severe liver disease, COPD Gold stage IV, coexisting conditions with life expectancy of less than a year, or congenital heart disease. The clinical study endpoints were hospitalization for HF, cardiac transplantation, left ventricular assist device implantation and mortality.

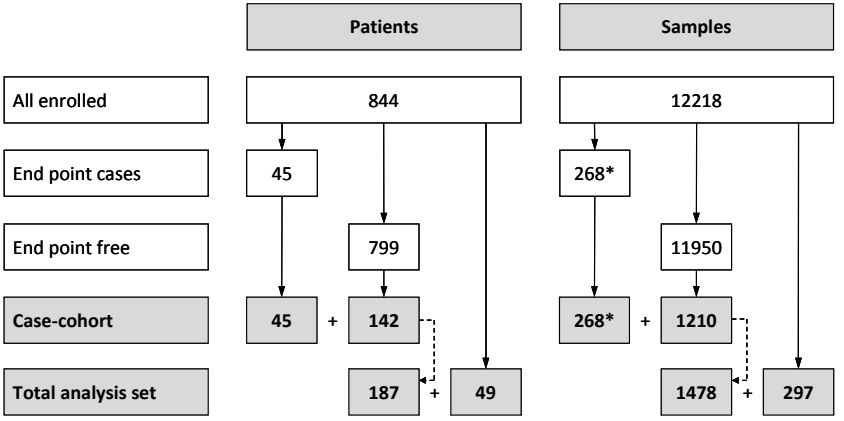

Figure 1.3 Display of patient selection in the BIOMArCS study and the total available number of blood samples.

After a first baseline assessment, patients were scheduled for study follow-up visits every 3 months with a maximum follow-up of 30 months. At baseline and at every visit both blood and urine samples were taken. In addition, in a subset of approximately 100 patients enrolled in Erasmus MC, besides the regular blood sampling, echocardiograms were made every 6 months. It is this specific subset that I have used for my research investigating the incremental predictive value of repeated echocardiograms. For my research investigating novel biomarkers in patients with chronic HF, data of 263 Bio-SHiFT patients (first inclusion round) were used.

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Part I

Variability of key blood biomarkers in patients with stable coronary artery disease

Chapter 2

Temporal evolution of serum concentrations of high-sensitivity cardiac troponin during 1 year after acute coronary syndrome admission

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Abstract

Background

Detailed insights in temporal evolution of high-sensitivity cardiac troponin (hsTn) following acute coronary syndrome (ACS) are currently missing. We aimed to describe and compare the post-ACS kinetics of hsTnI and hsTnT, and to determine their intra- and inter-individual variation in clinically stable patients.

Methods and results

We determined hsTnI (Abbott) and hsTnT (Roche) in 1507 repeated blood samples, derived from 191 ACS patients (median 8/patient) who remained free from adverse cardiac events during one year follow-up. Post-ACS kinetics were studied by linear mixed effect models. Using the samples collected in the 6-12 months post-ACS timeframe - patients were then considered to have chronic coronary syndrome (CCS) - we determined (differences between) the average hsTnI and average hsTnT concentration, and the intra-individual and inter-individual variation for both biomarkers.

Compared to hsTnT, hsTnI peaked higher (median 3506ng/L vs 494ng/L, $p<0.001$) and was quicker below the biomarker-specific upper reference limit (URL) (16 vs. 19 days, $p<0.001$). In the post 6 months samples, hsTnI and hsTnT showed modest correlation ($r_{spearman}=0.60$), whereas the average hsTnT concentration was 5 times more likely to be above the URL than hsTnI. The intra-individual variation of hsTnI and hsTnT were 14.0% and 18.1%, while the inter-individual variation were 94.1% and 75.9%.

Conclusions

HsTnI peaked higher after ACS and was quicker below the URL. In the post 6 months samples, hsTnI and hsTnT were clearly not interchangeable and average hsTnT concentrations were much more often above the URL than hsTnI. For both markers, the within-patient variation fell largely below between-patient variation.

Introduction

High-sensitivity cardiac Troponins (hsTn) are now widely used in clinical practice, and are key elements of the diagnosis of myocardial infarction (MI) in patients presenting with ischemic chest pain[1,2]. In the setting of suspected acute coronary syndrome (ACS), hsTnI and hsTnT have a comparable very good performance and are practically interchangeable[3]. However, hsTns are nowadays also measured for other purposes than diagnosing ACS, e.g. as part of perioperative care[4], and studies comparing hsTnI concentrations and hsTnT concentrations outside the setting of ACS are scarce and mostly performed in the general population[5,6].

In the current study, we utilized the 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) with high-frequency blood sampling[7-9], investigating in detail the evolution of hsTnI and hsTnT concentrations until one year after ACS admission. We aimed to describe (differences in) the post-ACS kinetics, and differences in the hsTnI and hsTnT concentrations after the biomarker reached stable levels. In addition, we explored the biological variation of cardiac troponins, measured with contemporary high-sensitivity assays.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study design

The study design and main results of BIOMArCS has been published previously[7-9]. In short, BIOMArCS is a multi-center, prospective, observational study that was conducted in 18 participating hospitals in the Netherlands during 2008-2015. The study was designed to obtain detailed data on biomarker patterns until one-year follow-up post-ACS. Patients above 40 years presenting with ACS and at least one additional cardiovascular risk factor were eligible for enrolment. Exclusion criteria were ischemia precipitated by a condition other than atherosclerotic chronic coronary syndrome (CCS), a left ventricular ejection fraction <30%, or end-stage congestive heart failure (NYHA class ≥3), severe chronic kidney disease with

measured or calculated glomerular filtration rate (Cockroft-Gault orMDRD4 formula) of <30 mL/min/1.73 m^2, or a coexistent condition with life expectancy <1 year. All patients were treated according to prevailing guidelines and at the discretion of the treating physician. The study protocol was approved by the Institutional Review Board of the participating hospitals, and all study subjects gave written informed consent.

Blood sampling and storage

Blood samples were collected at admission, at the day of hospital discharge and subsequently every fortnight during the first six months after discharge. If logistic circumstances hindered inclusion during hospitalization, patients could be included on the first outpatient visit within 6 weeks after discharge. In a subset of approximately 8% of patients, additional blood samples were collected within 24, 48, 72 and 96 hours after admission and at the day of hospital discharge with the specific aim to study the early evolution and normalization of the biomarkers. Follow-up was terminated permanently after coronary artery bypass grafting, hospital admission for heart failure, or a deterioration of renal function leading to a glomerular filtration rate <30ml/min/1.73 m^2.

Blood samples were handled and securely stored on-site. After preparation, aliquots were frozen at -80 degrees Celsius within two hours after withdrawal. Samples were transported under controlled conditions to the department of Clinical Chemistry at the Erasmus MC for long-term storage.

Study patients

For the BIOMArCS main results analysis we applied the case-cohort approach, including a total of 187 patients, of whom 45 reached the study endpoint of cardiovascular death or repeat ACS[7,8]. For the current analysis, we excluded these endpoint cases, and enriched the set with 49 patients who had daily sampling during the first 4 days after the index ACS. Hence, our analysis set consisted of 191 endpoint-free patients[7]. They contributed a median of 8 (25^{th}-75^{th} percentile 5-10) repeated serum samples per patient (altogether 1507 samples), in which hsTnI (Abbott) and a hsTnT (Roche) were determined in a blinded fashion and in one batch. These assays have a lower limit of detection (LLD) and population upper reference limit (URL; 99th percentile of the distribution in the general population)

of 1.2 ng/L and 26.6 ng/L for hsTnI, and 5 ng/L and 14 ng/L for hsTnT, respectively. The limit of blank was equal to the LLD for hsTnI and 3.0ng/L for hsTnT. Undetectable concentrations were assigned the concentration of 1.0 ng/L for hsTnI and 2.9 ng/L for hsTnT.

Data analysis

Continuous variables are presented as mean (standard deviation; SD) or median (25^{th}-75^{th} percentile), depending on their distributions. Categorical variables are summarized as numbers and percentages. Differences between hsTnI and hsTnT were investigated using McNemar's test for paired nominal data or a Wilcoxon signed rank test for paired continuous data.

Post-ACS kinetics

We used linear mixed effect (LME) models to describe the average cardiac troponin stabilization patterns over time. In these models, time was entered as the independent variable, and the log-transformed (because of the non-normal distribution) cardiac troponin value as the dependent variable. A total of two cubic splines were placed in order to model the non-linearity of the association between time and cardiac troponin concentration. We used Akaike's information criterion and Bayesian information criteria for the optimal placing of these splines. Random slopes as well as random intercepts were included in the models to allow for individual variation.

Using the fitted LME models, we calculated the average hsTnI and hsTnT concentrations on a day-to-day basis for each patient. These concentrations were then used to estimate the peak concentration, the time until peak concentration, the median time during which cardiac troponins were elevated above the population reference value after the index ACS, and the median time until stabilization. We defined stabilization as a difference in (model-derived) cardiac troponin concentrations of less than one percent between two consecutive days.

Measures of biological variation

For investigating the parameters of variability of a biomarker, it is necessary that the patients is in a (biochemically) stable status. Based on previous studies with repeated cardiac echoes and blood measurements, we presumed that hsTn

concentrations would be biochemically stable at 6 months post-ACS[10-12]. Accordingly, the analysis of biological variation was based on 446 samples (median 4 sample per patient (range 3-9) that were collected 6-12 months after the index ACS, and was limited to the 98 patients who had ≥3 measurements in that time window and who did not undergo a (staged) percutaneous coronary intervention (PCI) - thus iatrogenic distortion of the cardiac troponin concentrations caused by PCI was excluded[13].

We determined the coefficient of variation (CV) of hsTnI and hsTnT, and applied the method of Fraser and Harris [14] to split the total variation in 3 components. These represent the variation due to the imprecision of the analytical process (CV_a), the intra-individual or within-subject variation (CV_i) and the inter-individual or between-subject variation (CV_g). CV_a can be determined by repeatedly measuring the same sample using different assays. However, since this procedure is expensive, time-consuming and resource draining, laboratories generally use the CV_a that is based on a reference sample. We used the lab-specific CV_a of 5.0% for hsTnI and 3.0% for hsTnT, respectively. Besides determining the different coefficients of variability, we also calculated the Index of Individuality (II) and the Reference Change Value (RCV) for both biomarkers. The II is the ratio of the combined within-subject and analytical variation relative to the between-subject variation. Previously it has been suggested that in case of an II <0.6, individual subjects should have their own reference values instead of a population based reference [15]. When the II >1.4, a population-based reference is preferred. The RCV reflects the limit of (relative) change in biomarker values in individual subjects that can be explained by the combined within-subject and analytical variation. Finally, we investigated factors associated with the CV_i using linear regression. A more detailed description of the parameters of variability and the formulas used to calculate them are included in the *supplementary files*.

Patient-specific reference value

The average time until hsTnI and hsTnT stabilization after the index ACS appeared less than one month, whereas within-subject variability was relatively small. Therefore, we conducted a post-hoc analysis of all 122 patients with >3 samples in the >1 month time window to learn if a patient-specific reference value could be determined this early after the index ACS, as follows. We calculated the moving average of two consecutive hsTn measurements, which was then compared with

the next measurement. If the difference was less than 5 ng/L the moving average was then considered the patient-specific reference. The 5 ng/L threshold was chosen, since that value was equal to the median patient-specific hsTnT concentration times the upper limit of the RCV.

All analyses were performed using R 3.1.1 using packages 'nlme' [16] and 'splines'[17].

Results

Baseline characteristics

Baseline characteristics are presented in Table 2.1. The mean age of the patients in the analysis set was 63.0 (11.1) years and 78% were men. More than half of the population had hypertension (52.1%) and a large proportion had hypercholesterolemia (47.5%) and/or a family history of CCS (53.5%). ST-elevation myocardial infarction was the most common index event (46.2%), followed by non-ST-elevation myocardial infarction (40.7%). No relevant differences in baseline characteristics were identified when comparing the full analysis set with the patients used to determine biological variation.

Post-ACS kinetics

The average concentrations of the different biomarkers from the time of the ACS until day 50, are shown in Figure 2.1. Both hsTnI and hsTnT peaked on day 1 (median, interquartile range (IQR) 1-2) and gradually returned to concentrations beneath the population URL. The median peak concentration was 3506 ng/L (IQR 2300-6596) for hsTnI and 494 ng/L (397-939) for hsTnT ($p<0.001$). Although statistically significant, there was little difference in the median time until stabilization on patient-level. Median number of days were 31 (IQR 30-32) days for hsTnI and 30 (IQR 30-31) days for hsTnT ($p<0.001$), respectively. In contrast, hsTnI was quicker below the URL than hsTnT (median 16 (13-19) days vs. 19 (16-26) days, $p<0.001$).

Table 2.1 Baseline characteristics.

	Analysis set (n=191)	Post 6 months (n=98)
Age. Y (SD)	62.4 (10.6)	62.8 (9.5)
Male gender (%)	148 (77.5)	77 (78.6)
Cardiovascular risk factors (%)		
Diabetes Mellitus	33 (17.3)	17 (17.3)
Hypertension	101 (52.9)	52 (53.1)
Hypercholesterolemia	92 (46.5)	54 (58.2)
Family history of CCS*	87 (53.0)	47 (59.5)
Current smoker	80 (41.9)	41 (41.8)
History of cardiovascular disease (%)		
MI	50 (26.2)	30 (30.6)
CABG	14 (7.3)	6 (6.1)
PCI	44 (23.2)	28 (28.9)
Stroke	19 (9.9)	7 (7.1)
Admission diagnosis (%)		
STEMI	93 (49.0)	47 (48.0)
NSTEMI	74 (38.7)	37 (37.8)
UAP	24 (12.6)	14 (14.3)
Physical examination		
Body mass index (SD)	27.5 (3.6)	27.5 (3.6)
Killip class 1 (%)	177 (92.7)	94 (95.9)
Heart rate (IQR)	73 (62-84)	70 (61-81)
Systolic blood pressure (IQR)	137 (117-152)	136 (119-151)
eGFR, ml/min/1,73 m^2 (SD)	98 (30)	97 (28)
Medication (%)		
Aspirin	183 (96.3)	95 (96.9)
BetaBlocker	167 (87.9)	83 (84.7)
ACEi	138 (72.6)	68 (69.4)
ARB	22 (11.6)	11 (11.2)
Statin	183 (96.3)	96 (98.0)

SD: Standard deviation; IQR: Interquartile range; Y: year; CCS: chronic coronary syndromes; eGFR: estimated glomerular filtration rate; MI: Myocardial infarction; CABG: coronary artery bypass grafting; PCI: percutaneous coronary intervention; STEMI: ST-elevation myocardial infarction; NSTEMI: non ST-elevation myocardial infarction; UAP: unstable angina pectoris. *Family history of CCS was defined as defined as angina pectoris, myocardial infarction or sudden abrupt death without obvious cause, before the age of 55 in a first-degree blood relative. Post 6 months: Analysis set minus (1.) an elective PCI more than 150 days after the index event and (2.) patients with less than 3 samples available after 6 months.

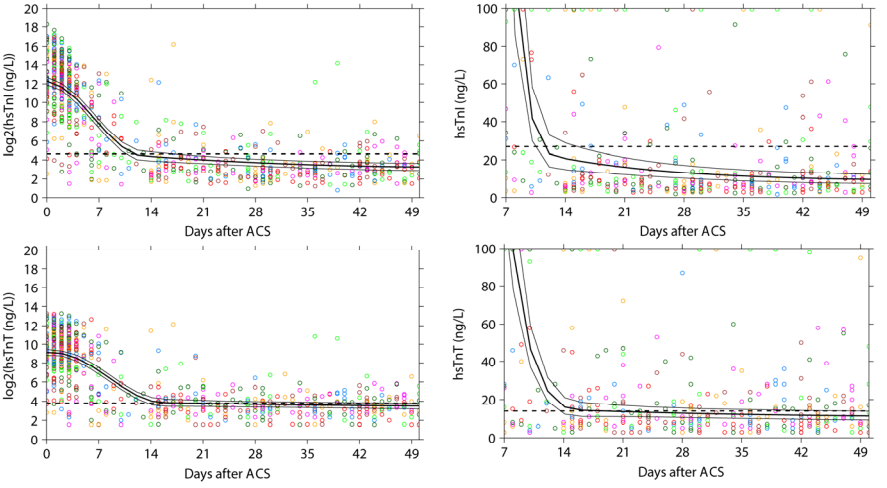

Figure 2.1 Average stabilization patterns of high-sensitivity cardiac troponins after ACS. The X-axes depict the number of days since the ACS. The Y-axes represent the cardiac troponin concentrations. The left two plots are on the log scale with base number 2. A 1 point increase can thus be interpreted as a doubling of the value. The black lines depict the cohort average; the dashed lines the corresponding 95% confidence interval.

Biological variation

Figure 2.2 depicts all pairs of hsTnI and hsTnT measurements taken after 6 months. All hsTnI values exceeded the LLD, whereas 22.0% of hsTnT values were below the LLD (9.0% below the limits of blank). In all the samples, 2.0% of hsTnI and 17.2% of hsTnT values exceeded the population URL ($p<0.001$); 3 patients had an average hsTnI above the URL compared to 16 patients with an average hsTnT above the URL ($p=0.002$). The Spearman correlation for average hsTn level was $r=0.60$ ($p<0.001$).

The distributions of the hsTn measurements after 6 months are shown for each patient in Figure 2.3. CV_is of hsTnI and hsTnT were 14.0% and 18.1%, respectively. We could not identify any baseline characteristics that were significantly associated with the observed CV_is (Supplementary Table S2.1). In contrast to the small CV_is, the CV_gs were large, reflecting relatively large differences in average cardiac troponin concentrations between patients. Consequently, both biomarkers had II's <0.6, The RCV limits ranged between -33.6% and 50.5% for hsTnI, and -39.6% and

65.5% for hsTnT, respectively. Consequently, as an example, in a patient with a steady state hsTnI concentration of 5 ng/L, a rise of 3 ng/L exceeds the combined analytical and within-subject variation with 95% certainty, and can thus be considered the consequence of pathological processes. An overview of the different parameters of biological variation is presented in Table 2.2.

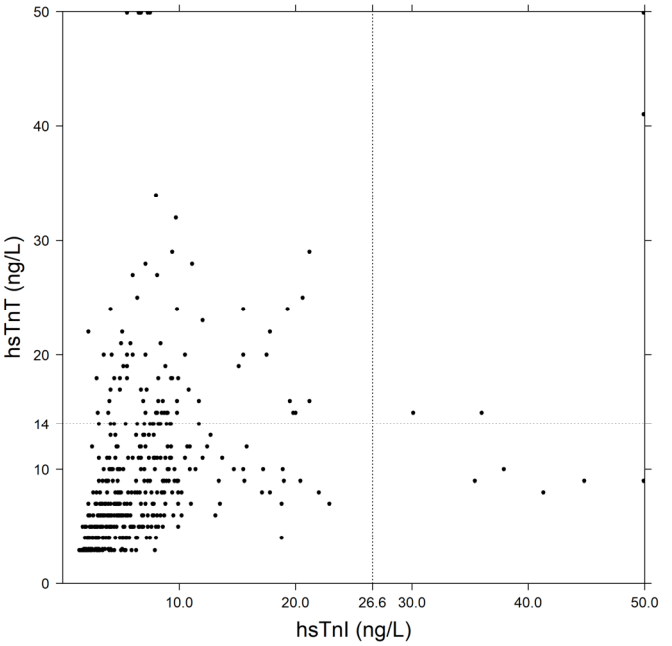

Figure 2.2 Comparision of high-sensitivity cardiac troponin I and T concentrations in the samples taken after six months. The X-axis depict the hsTnI concentration while on the Y-axis the concentration of hsTnT is given. Each dot represents a single blood samplein which thus both a hsTnI and hsTnT concentration has been measured. hsTnI: high-sensitivity cardiac troponin I; hsTnT: high-sensitivity cardiac troponin T.

Patient-specific reference value

In the post-hoc analysis of 122 patients (see the Methods section), a patient-specific reference value could be determined in 85.2% (hsTnI) and 83.6% (hsTnT) using the first two post 30-day measurements. The median (25^{th}-75^{th} percentile) reference values were 7.1 ng/L (4.4-10.6) and 8.5 ng/L (6.5-12.9) for hsTnI and

hsTnT, respectively. The difference between the patient-specific baseline value and their last available measurement (on average 11 months after the index ACS) was less than 5 ng/L in more than 81.7% (hsTnI) and 77.5% (hsTnT) of the patients. A paired t-test confirmed that there were no significant differences between the patient-specific baseline value based on the first two measurements, and the last available measurement for both hsTnI (mean difference -.37 ng/L (95% confidence interval -3.26-2.53, p=0.80) and hsTnT (mean difference 0.11 ng/L (95% confidence interval -1.81–2.03, p=0.91).

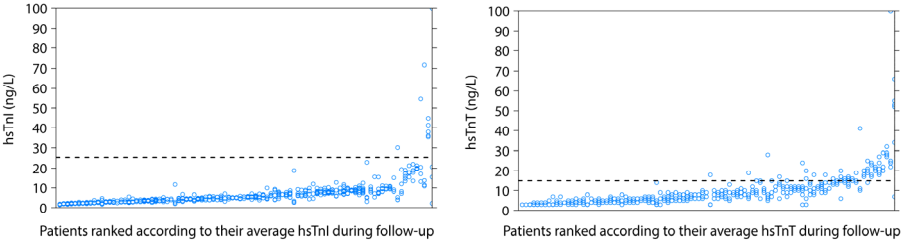

Figure 2.3 Distribution of the high-sensitivity cardiac troponins after six months. On the horizontal axes are the individual patients ranked based on their average cardiac troponin values. The vertical axes depict the cardiac troponin concentrations resulting from the repeated measurements. The dotted lines show the reference value of the cardiac troponi.

Table 2.2 Overview of parameters of biological variation.

	Average patient concentration (ng/ml)	CVa (%)	CVi (%)	CVg (%)	II	RCV (%)	Log-normal	
							RCV low (%)	RCV up (%)
HsTnI	5.3 (3.7-8.3)	5.0	14.0	94.1	0.16	38.7	-33.6	50.5
HsTnT	7.8 (5.1-11.1)	3.0	18.1	75.9	0.24	50.1	-39.6	65.5

HsTnI: high-sensitivity cardiac troponin I; HsTnT: high-sensitivity cardiac troponin T; CVa: analytical coefficient of variation; CVg: interindividual coefficient of variation; CVi: intraindividual coefficient of variation; II: index of individuality; RCV: reference change value.

Discussion

In BIOMArCS, we confirmed the hsTn peak, the plateau after the index ACS and that values can remain above the population URL for a prolonged time[18]. We added that after a quick decrease, the median time to reach values below the URL was

shorter for hsTnI than hsTnT. In addition, post-6 months samples in (then) stable CCS patients, the percentage of hsTnT measurements with concentration above the population URL was far greater than that of hsTnI (Figure 2.3) with (thus) poor interchangeability of the two biomarkers. The individual variation of both hsTnI and hsTnT were low, while differences between patients were large. This combination of characteristics led to a low II (<0.6) for both cardiac troponins, which again stresses that in patients with known stable CCS after having previously endured an ACS, patient-specific reference values are to be preferred over the population-based reference[15]. Finally, we were able to demonstrate that the patient-specific reference value can already be obtained based on two consecutive samples taken after one month in the vast majority of post-ACS subjects.

In our study, we found some striking differences between hsTnI and hsTnT. After the index ACS, hsTnI showed a higher peak concentration and had a quicker decent when compared to hsTnT. The higher peak levels had been previously described by Laugaudin et al in 106 consecutive ST-elevation MI patients[19]. We now add to this that hsTnI is also faster below the population URL than hsTnT. After 6 months, when patients were to be considered biochemical stable, there were more than 5 times as much patients with an average hsTnT concentrations above the population URL than patients with hsTnI above the population URL. Moreover, despite statistically significant, the correlation between average hsTnI and hsTnT concentration clearly showed that the two markers cannot be considered interchangeable in an asymptomatic post-ACS population. Although obvious differences in design (single measurement versus multiple measurements) and participants (general population vs. ACS patients) are to be acknowledged, our findings are much in line with previous reports from general population cohorts comparing hsTns. In a study by Kimenai et al among 1540 individuals without significant baseline disease, the correlation coefficient between hsTnI and hsTnT was 0.55[6], while among 19501 participants of the General Scotland Scottisch Family Health Study the r was 0.46 [5]. Remarkably, in the latter study, the number of patients above the population URL was much greater for hsTnT than for hsTnI, which is in line with our results. We add to this current body of evidence that also in patients with known CCS the correlation between hsTnI and hsTnT concentrations are not strong.

To date, studies on the biological variation of cardiac troponins, measured with contemporary high-sensitivity assays, are scarce and their sample sizes have usually

been small[20-24]. Particularly in patients with established CCS, such as post-ACS patients, little to no information is available. The parameters of variation found in our study are comparable to earlier reports in subjects sampled from the general 'healthy' population. For example, Wu et al. reported a long-term individual variation of 14% for hsTnI, based on 17 healthy subjects[23]. The CV_g in their report was lower than in our study, which suggests that cardiac troponins show larger variations in CCS patients than in healthy individuals. The larger between-subject variation in a diseased population compared to a healthy one, is also confirmed by a study of Meijers et al. comparing biological variation in 83 patients with heart failure to 28 healthy subjects[25]. They reported a CV_g for hsTnT of 96.6% and 51.2% respectively. The CV_is however, were similar in both populations and comparable to our cohort.

We were able to demonstrate the feasibility of obtaining patient-specific references values in patients with established CCS. This reference value could be retrieved in the majority of our post-ACS patients based on a limited number of consecutive measurements, whereas these values showed good agreement with samples taken later during follow-up. It is our opinion that the patient-specific reference value can help fine-tune the diagnostic process in specific situations. These reference values could be of help to fine-tune a personalized approach in post-ACS patients, in particular in those with asymptomatic elevations that were found by chance (e.g. hsTn measurements in the perioperative setting), and in those presenting with unclear symptoms. For instance, if a patient comes with atypical complaints and has slightly elevated hsTn concentrations in two consecutive measurements. Atypical presentations are not uncommon[26] and a rise of hsTns concentrations cannot always be identified, particularly if patients come several hours after the complaints start when cardiac troponin levels might already be in the plateau phase. Comparing the hsTn concentrations measured with the patient-specific reference could help determine if this patient is more likely to have an ACS and needs to go to the Cath lab or can be sent home. Also, when a patient has typical complaints but the hsTn concentrations are still below population URL with a borderline rise between the two consecutive measurements comparing the concentration with their individual reference value might determine the final decision. If the found concentration is (much) higher than the patient-specific reference value (but still below population URL) than it is probably more likely to be unstable angina pectoris or an MI. Accurately diagnosing unstable angina is

important as these patients often need early PCI and have an incidence rate of future (lethal) cardiac event comparable to patients that had a non-ST elevation MI[27,28]. Moreover, particularly when using hsTnI, MI is known to be underdiagnosed because of the relatively high population URL[29].

Limitations

The high-frequency blood sampling design of BIOMArCS enables an in-depth analysis of longitudinal biomarker patterns in population of patients with established CCS. A limitation of the current analysis is that compared to an real-world ACS population such as the SWEDEHEART registry[30], the subjects included in the current study are on average 8 years younger, were more likely to have a ST-elevation MI (49% vs. 35.5%), had more previous PCI's performed (29.1% vs. 13.8%) and had a lower prevalence of diabetes mellitus (17.3% vs. 22.5%). These differences might compromise the generalizability of the results. Moreover, the generalizability of our parameters could potentially be further compromised as per study protocol, we excluded all patients with recurrent events during the year follow-up as we did not want to take into account possible distortion from an imminent ischemic event while calculation the parameters of variability. However, in a sensitivity analysis also comprising the patients with ischemic events, the parameters only changed marginally (data not shown). Secondly, information on the patient's activities prior to sampling is lacking and that the timing of blood sampling during the day was not specified. HsTns are known to be influenced by (heavy) physical activity[31] and hsTnT, but not hsTnI, is known to exhibit a diurnal rhythm[32]. However, we have investigated the variation of the time of sampling, and found that all measurements were taken between 8 o'clock in the morning and 4 o'clock in the afternoon. Moreover, we observed that, although not specified in the protocol, the vast majority of the patients had repeated visits for blood sampling at the same hour of the day. Hence, the within-patient variation in biomarker concentrations found in this study, cannot be explained by variations in sampling time. Thirdly, no echocardiographic data are available which could have been an aid in explaining chronic elevated cardiac troponin concentrations in different patients. A final limitation is that using our data, although plausible we cannot confirm that using a patient-specific reference value enhances the diagnostics for future ACS. This should be the focus of future research.

Conclusion

In conclusion, hsTn concentrations showed similar post-ACS kinetics however, after the initial peak, hsTnI had a quicker median time to concentrations below population URL than hsTnT. In the post 6 months samples, hsTnI and hsTnT showed modest correlation ($r_{spearman}$=0.60), whereas the average hsTnT concentration was 5 times more likely to be above the URL than hsTnI. The within-patient variation was small for both cardiac troponins, and comparable to healthy populations. Between-patient variation, however, is much higher in post-ACS patients than in population controls. Consequently, our data supports the use of patient specific reference values for hsTn in CCS patients. Patient-specific reference values can easily be obtained in the vast majority of patients by using two consecutive samples during a clinically stable phase

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Chapter 3

Stabilization patterns and variability of hs-CRP, NT-proBNP and ST2 during one year after Acute Coronary Syndrome admission: results of the BIOMArCS study

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Abstract

Introduction

Details of the biological variability of high sensitivity C-reactive protein (hs-CRP), N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and ST2 are currently lacking in patients with acute coronary syndrome (ACS) but are crucial knowledge when aiming to use these biomarkers for personalized risk prediction. In the current study, we report post-ACS kinetics and the variability of the hs-CRP, NT-proBNP and ST2.

Methods

BIOMArCS is a prospective, observational study with high frequency blood sampling during one year post-ACS. Using 1507 blood samples from 191 patients that remained free from adverse cardiac events, we investigated post-ACS kinetics of hs-CRP, NT-proBNP and ST2. Biological variability was studied using the samples collected between 6-12 months after the index ACS, when patients were considered to have stable coronary artery disease.

Results

On average, hs-CRP rose peaked at day 2 and rose well above the reference value. ST2 peaked immediately after the ACS but never rose above the reference value. NT-proBNP level rose on average during the first 2 days post-ACS and slowly declined afterwards. The within-subject variation and relative change value (RCV) of ST2 were relatively small (13.8%, RCV 39.7%), while hs-CRP (41.9%, lognormal RCV 206.1/-67.3%) and NT-proBNP (39.0%, lognormal RCV 185.2/-64.9%) showed a considerable variation.

Conclusion

Variability of hs-CRP and NT-proBNP within asymptomatic and clinically stable post-ACS patients is considerable. In contrast, within-patient variability of ST2 is low. Given the low within-subject variation, ST2 might be the most useful biomarker for personalizing risk prediction in stable post-ACS patients.

Introduction

Elevated serum levels of high sensitivity C-reactive protein (hs-CRP), N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and soluble ST2 (ST2) have been associated with adverse cardiovascular events in patients with coronary artery disease (CAD) and acute coronary syndrome (ACS), and have been proposed in prognostic models[1-8]. However, the differences in serum levels between the patients with and without cardiovascular events are often not large. For example, in a study by Zebrack et al. among 2554 patients undergoing coronary angiography, in the group without CAD and the lowest event rate during a mean follow-up of 2 years median CRP levels was from 1.15 mg/dL, compared median levels of 1.28mg/dL in the group with the most severe CAD and highest event rate during follow-up[4].

While aiming for personalized risk prediction, appropriate stratification of patients is crucial. Thus, it is important to know if differences in biomarkers levels between subjects, and changes over time within a patient, truly reflect differences in health state, or if it is caused by analytical or by biological variability. Studies on the variability of hs-CRP, NT-proBNP and ST2 during stable health have mostly been performed in (small sets of) healthy subjects, or in heart failure patients[9-18]. Remarkably, data on their performance in stable post-ACS/CAD patients is scarce[19].

Against this background, we aimed to provide a detailed description of the influence of an ACS on hs-CRP, NT-proBNP and ST2 levels, and to investigate the within- and between-patient variability of these biomarkers in serial blood samples during stable health after ACS. Our analyses are embedded in the BIOMarker study to identify the Acute risk of a Coronary Syndrome (BIOMArCS), which was specifically designed to study longitudinal biomarker patterns in (post-)ACS patients[20].

Methods

BIOMArCS is a multi-center, prospective, observational study that was conducted in 18 participating hospitals in the Netherlands during 2008-2015. The study was designed to obtain data on biomarker patterns in ACS patients during one-year

follow-up. Details of the BIOMArCS design and main findings have been published previously[20-22].

Briefly, patients above 40 years presenting with ACS and at least one additional cardiovascular risk factor were eligible. Preferably, patients were enrolled during hospital admission, but inclusion at the first outpatient visit post-discharge (usually 4-6 weeks later) was allowed. Blood samples were collected at admission, at the day of hospital discharge and subsequently every fortnight during the first six months after discharge. Additional blood samples were collected at 24, 48, 72 and 96 hours after admission and at the day of hospital discharge in a subset of 8% of patients, with the specific aim to study the evolution and normalization of biomarkers in the early post-ACS phase. Follow-up was terminated permanently after coronary artery bypass grafting, hospital admission for HF, or a deterioration of renal function leading to a glomerular filtration rate <30 ml/min/1.73 m^2, as circulating biomarker concentrations may be significantly influenced by these conditions.

All patients were treated to prevailing guidelines and at the discretion of the investigator. The study was approved by the medical ethics committees and conducted in accordance with the Declaration of Helsinki. All patients signed informed consent for their participation in the study.

Blood sampling and storage

Blood samples were handled and securely stored on-site within 4 hours after venipuncture. After preparation, aliquots were frozen at -80 degrees Celsius within two hours after withdrawal. Samples were transported under controlled conditions to the department of Clinical Chemistry at the Erasmus MC for long-term storage. After all material was collected and follow-up was completed, batch wise analysis of blood samples was performed in a central laboratory. Laboratory personnel was blinded for patient characteristics.

Biomarker measurements were performed in the serum EDTA plasma after a median average storage time of 4.9 (25th-75th percentile 3.8-6.2) years. Hs-CRP was determined using the Coulter 5800 series (Beckman Coulter, Brea, California, USA), lower limits of detection (LLOD) 0.2 mg/L, and population reference value 5 mg/L. ST2 was determined with the Presage ST2 assay (Critical diagnostics, San

Diego, California, USA), LLOD 1.31 ng/mL, and reference value 49.3 ng/mL (male) or 33.5 ng/mL (female). NT-proBNP was measured with a custom-built ELISA method using an antibody against HRP-conjugated MAB mouse anti-human N-terminal proBNP (Hytest, 13G12 (4NT1C)), which shows very good agreement with other commercially available assays. The intra-assay CV was 4%, LLOD 6.25 pmol/L, and reference value 30 pmol/L.

Analysis of the biomarker stabilization patterns

For the analysis of the BIOMArCS study, hs-CRP, NT-proBNP and ST2 serum levels were measured in the samples of 187 patients[21]. Of these 187 patients, 45 had a new ischemic event during the follow-up. For the current analysis, we removed the patients with a new ischemic event from the analysis set and enriched the set with 49 patients who had daily sampling during the first 4 days of the index ACS submission. Hence, our analysis set consisted of 191 endpoint-free patients. They contributed a median of 8 (25th-75th percentile 5-10) repeated samples per patient (altogether 1507 samples) that were used for the analysis of stabilization patterns.

We used linear mixed effect (LME) models to describe biomarker stabilization patterns over time. A maximum of two cubic splines were placed to model a possible non-linear evolvement. Mean values of hs-CRP, NT-proBNP and ST2 at each post-ACS day were then determined using the fitted LME models. The biomarker was considered stabilized when the difference in mean level between two consecutive days was less than one percent.

Measures of biological variability

A coefficient of variability (CV) of a series of measurements is defined as 100% times the standard deviation (sd) of the measurements divided by their mean value (X): $CV=100\% * sd/X$

According to the methods by Fraser and Harris [23], the total variability of a series of repeated measurements in individual subjects can be split in 3 components, which represent the variability due to the imprecision of the analytical process (CV_A), the intra-individual or within-subject variability (CV_i) and the inter-individual or between-subject variability (CV_g). Besides these measures of variability, we also determined the index of individuality (II) and the reference change value (RCV). The

RCV reflects the limit of (relative) change in biomarker values in individual subjects that can be explained by the combined within-subject and analytical variation while the II is calculated for investigating if population-based reference values are adequate. A more detailed description of the different measures of variability and the formulas used to calculate them can be found in the supplementary files.

Based on previous studies investigating cardiac remodelling and biomarker levels post-ACS, we presumed that ACS patients would be biochemically stable after 6 months[1,24,25]. Hence, for the analysis of biological variability, those patients that had ≥3 measurements in the 6-12 months post-ACS time window were selected. This resulted in a total of 446 samples and was limited to 98 patients.

We performed sensitivity analyses, investigating if the biological variation was influenced by the New York Heart Association (NYHA)-classification and Canadian Cardiovascular Society (CCS) grading. NYHA class and CCS grade were determined at all sampling moments. In our sensitivity analyses, we calculated the measures of biological variation while excluding patients who reported an elevated NYHA-class (NYHA ≥1) and/or elevated CCS grading (CCS ≥1) at any sampling moment.
All statistical analyses were performed with R 3.3.1. P-values below 0.05 (2-sided) were considered statistically significant.

Results

Patient characteristics

The mean age (standard deviation) of the patients was 62.4 (10.6) years and 78% were men (Table 3.1). A substantial percentage of patients had hypertension (53%), hypercholesterolemia (48%), and a family history of premature CAD (53%). ST-elevation myocardial infarction was the most common index event (49%). No relevant differences in baseline characteristics were identified between the two analysis sets.

Table 3.1 Baseline characteristics.

	Stabilization pattern set (n=191)	Biological variation set (n=98)
Age, year (SD)	62.4 (10.6)	62.8 (9.5)
Male gender, n (%)	148 (77.5)	77 (78.6)
Cardiovascular risk factors, n (%)		
Diabetes Mellitus	33 (17.3)	17 (17.3)
Hypertension	101 (52.9)	52 (53.1)
Hypercholesterolemia	91 (47.6)	53 (54.1)
Family history of CAD	87 (53.0)	47 (59.5)
Current smoker	80 (41.9)	41 (41.8)
History of cardiovascular disease, n (%)		
MI	50 (26.2)	30 (30.6)
CABG	14 (7.3)	6 (6.1)
PCI	44 (23.2)	28 (28.9)
Stroke	19 (9.9)	7 (7.1)
Admission diagnosis, n (%)		
STEMI	94 (49.2)	48 (49.0)
NSTEMI	72 (37.7)	36 (36.7)
UAP	25 (13.1)	14 (14.3)
Physical examination		
Body mass index, (SD)	27.5 (3.6)	27.5 (3.6)
Killip class 1, n(%)	177 (89.4)	94 (95.9)
Heart rate, (IQR)	73 (62-84)	70 (61-81)
Systolic blood pressure, (IQR)	137 (117-152)	136 (119-151)

CABG: coronary artery bypass grafting; CAD: coronary artery disease; IQR: interquartile range; MI: myocardial infarction; n: number; PCI: percutaneous coronary intervention; SD: standard deviation; STEMI: ST-elevation myocardial infarction; UAP unstable angina pectoris; KILLIP.

Stabilization patterns

The average stabilization patterns of the three biomarkers of interest in the post-ACS period are shown in Figure 3.1. Hs-CRP increased until day 2, and reached on average a maximum level of 14.9 mg/L. Thereafter, hs-CRP steadily declined. The population reference value was reached at day 15, and the marker had stabilized at day 30. NT-proBNP also increased until day 2, where it reached an average maximum level of 94 pmol/L. NT-proBNP only slowly declined. The marker stabilized at day 15, but levels remained on average above the population reference value during follow-up. ST2 showed on average a maximum levels of 44.3 ng/mL at the day of the index ACS, which was well below the population reference value. Although still slowly declining, serum levels stabilized at day 5.

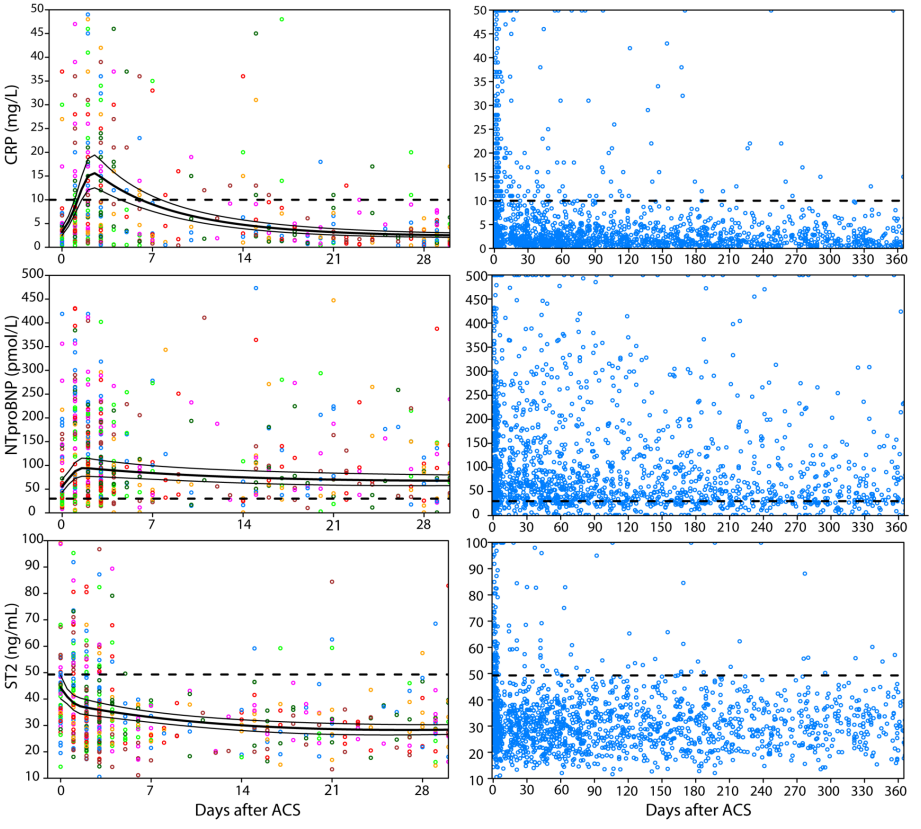

Figure 3.1 Temporal patterns of hs-CRP, NT-proBNP and ST2 after ACS. Left pictures depict the washout pattern after the ACS, the right pictures show all measurements during the year of follow-up. hs-CRP: high sensitivity C-reactive protein; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; ST2: Soluble suppression of tumorigenicity-2.

Biological variation

The median patient average serum levels in the 6-12 months post-ACS period are 2.4 mg/L (IQR 1.2-3.1) for hs-CRP, 54.4 pmol/L (IQR 29.1-97.8) for NT-proBNP and 30.2 ng/mL (IQR 25.2-35.0) for ST2. The distribution of the hs-CRP, NT-proBNP and ST2 measurements in the 6-12 months post-ACS period are shown for each patient in Figure 3.2. All hs-CRP and ST2 measurements were above the LLOD. NT-proBNP was

below the LLOD in 7.2% of the samples. Hs-CRP values were above the population reference in 15.5% of the samples, NT-proBNP in 24.1%, and for ST2 in 3.5%.

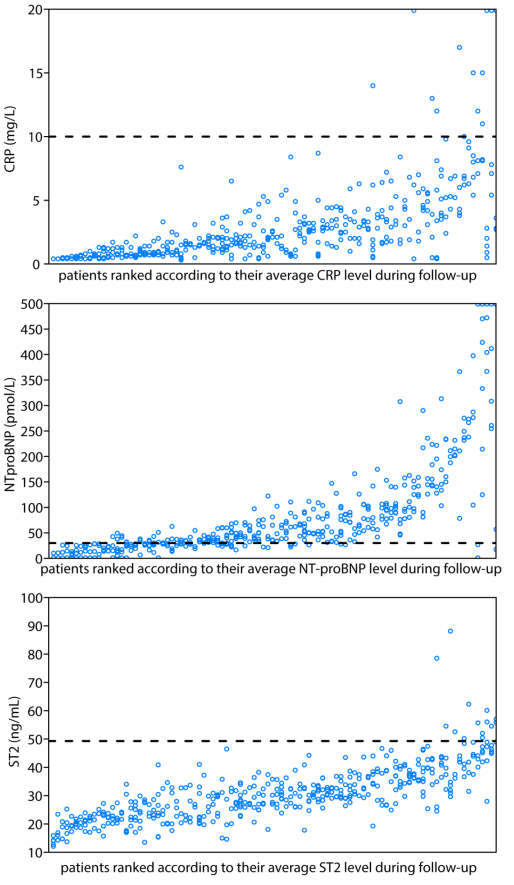

Figure 3.2 Distribution of measurements per patient. Horizontal: patients ranked according to their average biomarker value. Vertical: Spread of biomarker measurement per patient. hs-CRP: high sensitivity C-reactive protein; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; ST2: Soluble suppression of tumorigenicity-2.

Hs-CRP (CV_i 41.9%, lognormal RCV 206/-67%) and NT-proBNP (CV_i 39.0%, lognormal RCV 185/-65%) displayed a considerable within-individual variation and correspondingly wide RCVs, while the plasma concentrations of ST2 within a

patient were rather stable (CV_I of 13.8%, RCV 40%). The within-subject variability of hs-CRP (Kruskal-Wallis, p=0.36) and ST2 (p=0.17) was not influenced by the patients average serum levels. In contrast, the within-subject variation of NT-proBNP (Kruskal-Wallis, p=0.003) was much larger in patients with low serum concentrations (Figure 3.3). All three studies biomarkers had an II below 0.6, indicating that a patient-based reference value, based on previous samples of the individual patient is preferred. A detailed overview of the parameters of variation is shown in Table 3.2.

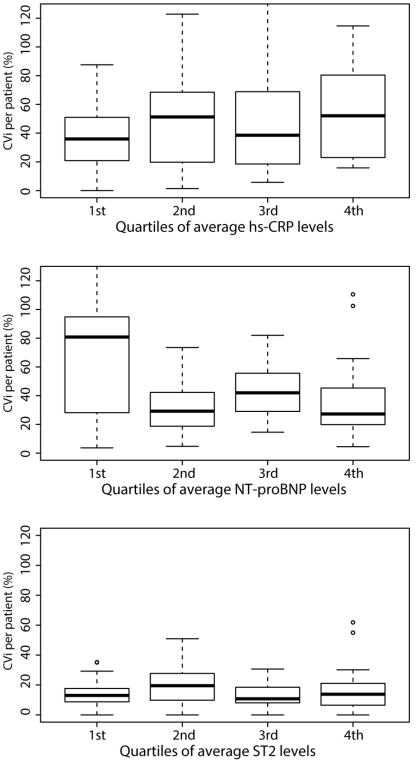

Figure 3.3 Intra-individual variability in quartiles based on average biomarker level. Boxplots of individual CV_Is in the different biomarker quartiles. hs-CRP: high sensitivity C-reactive protein; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; ST2: Soluble suppression of tumorigenicity-2. Median [range] of quartiles: Hs-CRP 1st: 0.8 [0.4, 1.2] 2nd: 1.8 [1.3, 2.4], 3rd: 3.0 [2.6, 3.5], 4th: 5.1 [3.6, 21.3]; NT-proBNP 1st: 20 [3, 29] 2nd: 39 [29, 54], 3rd: 66 [55, 91], 4th: 174 [100, 783]; ST2: 1st: 22.2 [13.8, 25.2] 2nd: 27.3 [25.3, 30.2], 3rd: 31.4 [30.2, 34.9], 4th: 41.2 [35.0, 54.0].

Sensitivity analyses

A NYHA class ≥1 was reported at 29 sampling moments (6%) in 15 different patients, while a CCS ≥1 was reported at 49 (11%) sampling moments in 27 different patients. In the majority of the cases, this concerned NYHA class and CCS class one (44.8% and 75.5% respectively). The coefficients of variation calculated in the dataset excluding these patients, showed similar results as the full cohort (Supplementary Table S3.1).

Discussion

Levels of hs-CRP, NT-proBNP and ST2 appeared differently affected by an ACS. Both Hs-CRP and NT-proBNP reached maximum values at day two, however, hereafter hs-CRP declined to levels below the population reference within two weeks, while the NT-proBNP only slowly declined and remained above the population reference value throughout the follow-up. ST2 was elevated at the time of the index ACS, but values remain below the population reference. Hs-CRP and NT-proBNP showed substantial within-subject variability and thus wide RCV, while the within-subject variability in of ST2 measurement was low. The between-subject variability was much larger than the within-subject variability for all three biomarkers.

Hs-CRP is one of the most used biochemical marker of inflammation in medicine and is known to rise after ACS due to inflammation of the ischemic areas of the heart. In agreement with our findings, Orn et al. described a delayed rise of CRP and a relatively fast near-normalization hereafter in 42 STEMI patients[26]. Similarly, among 962 patients with an episode of unstable CAD (NSTEMI and UAP), a peak in CRP serum concentration at 48 hours after the start of symptoms was described. In this same population the CRP levels at six months was on average still elevated when compared to healthy controls, although not above the reference value[27]. We now show that although the CRP levels are quickly within the “normal” range, it can take much longer before the levels actually stabilize.

Details of the parameters of variability of hs-CRP had not yet been described in a post-ACS population. However, in healthy volunteers, the within-subject variability is known to be considerable while the between-subject variability is even larger[9-11]. In our post-ACS patients, we found comparable within-subject variability and RCVs

as reported in healthy populations, but the variation between post-ACS patients appeared much larger. Given the high within-patient variability, it would take numerous numbers of samples to determine the habitual value needed to use CRP in personalized risk prediction in clinically stable CAD patients. This makes sense, as hs-CRP is not a specific cardiac marker and can be influenced by many other factors.

NT-proBNP showed an initial rise and maximum value at day 2 followed by a slow decline thereafter, with the levels remaining above the population reference value. The early rise can be explained by the initial myocardial ischemia[28], while the slow decline is most likely caused by progressive remodelling combined with a degree of myocardial dysfunction post-ACS[29]. However, as repeated cardiac imaging was not part of our study protocol, we cannot confirm this. The post-ACS kinetics of NT-proBNP have previously been described by Taiwar et al. and Lidahl et al. in respectively 60 patients and 1216 myocardial infarction patients. Similar to our study, they described a peak of the biomarker serum levels in the first 48 hours after the index event and a slow decline hereafter[30,31]. Other investigations – using few samples taken weeks/months apart from each other and not specifically focusing on post-ACS kinetics –, also showed that the biomarker had a peak early after ACS, and only slowly declined between blood samples hereafter[1,2]. Our study distinguishes itself from previous studies by three key elements: our study is conducted in the contemporary PCI-era; we systematically obtained a median of 4 (IQR 4-5) samples per patient at regular time points during 1-year follow-up; we applied state-of-the-art statistical methods, including LME models, in order to account for intra-patient correlation of consecutive measurements.

The biological variability of NT-proBNP in patients with CAD has been described earlier by Nordenksjold et al. in a total of 24 patients[19]. Using two samples taken a median of 23 (IQR 4-58) days apart, they found a CVi of 20.4 with a log-normal RCV of +76/-43%. We obtained a larger sample of patients and applied a higher blood sampling frequency. Also, we enrolled a homogeneous series of patients who were admitted for ACS, whereas Nordenksjold et al. studied patients undergoing coronary angiography, of whom only 50% patients ultimately underwent revascularization. These differences in study design could easily explain the differences in variability, and the corresponding RCVs found. The variability of NT-proBNP has also been investigated in healthy subjects and heart failure patients. Similar to our study results, in all studies previously performed, NT-proBNP serum

levels consistently show considerable within-patient variability and large corresponding RCVs[12-15]. Because of this variability, a single NT-proBNP measurement does not suffice for determining the habitual value, and thus, also not for an adequate personalized risk prediction. Notably, the between-subject variability that we found was comparable to the heart failure patients (CV_g of 116.3%) and larger than the healthy subjects (54.0%) that were described by Meijers et al.[15]. This can probably be explained by the larger heterogeneity in health status among patient populations when compared to healthy populations.

The early post-ACS evolvement of serum ST2 has been described based on 403 NSTE-ACS patients who participated in GUSTO-IV, using blood samples at 24, 48 and 72 hours after inclusion.[5] Similar as in our study, ST2 reached its maximum during the first sample and quickly declined hereafter. Our results add to this that, once stabilized, ST2 is a very stable marker with little variation over time in post-ACS patients. This is in line with previous studies investigating the biological variability of ST2, that all showed little within-patient variation and thus relatively small RCVs. Both Wu et al. and Dieplinger et al. report a CV_i of reported a CV_i of approximately 10% in small sets of healthy subjects[17,18], which was similar as the CV_i in series of chronic heart failure patients[15,16]. Interestingly, in the study by Meijers et al. the between-subject variability of ST2 in HF patients did not differ much from healthy controls (36.9% vs. 30.4%)[15]. Given the promising results of ST2 as a prognostic marker in patients with ACS and/or CAD[5-8], and the low within-patient variability of serum ST2 levels in post-ACS, a single, or a few, measurements would most likely improve personalized risk prediction.

Limitations

The BIOMArCS study provides us a unique platform to investigate the effect of ACS on the different blood biomarker and to investigate their parameters of variability in clinically stable post-ACS patients. However, a few limitations of our work need discussion. Blood sampling in BIOMArCS was protocolized, but the exact sample moment on the day was not. Consequently, differences in physical activities and diet, as well as potential circadian variation could have influenced the measures of biological variation[32-34]. Still, importantly, all samples were taken between 8am and 4pm, whereas the vast majority of patients had their blood sampling at the same time, which, apparently best fitted in their private schedule. Secondly, as we used

one central laboratory for the analysis of the blood samples, we could not investigate variability between different laboratories.

Conclusion

In conclusion, the within-patient variability of hs-CRP and NT-proBNP within asymptomatic and clinically stable post-ACS patients is substantial. This leads to clinically significant differences between serial measurements in the same patients. If used for personalized risk prediction, this would compromise the calibration and multiple samples would be needed in order to correctly classify the patients in the right risk category. In contrast, within-patient variability of ST2 is low. Given the low within-subject variation, ST2 might be the most useful biomarker for personalized risk prediction in stable post-ACS patients.

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Chapter 4

Variability of lipid measurements can have major impact on treatment during secondary prevention

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Research letter

Lowering low-density lipid cholesterol (LDL-C) is a vital part of secondary prevention in acute coronary syndrome (ACS) patients. The AHA/ACC 2018 guideline advocates to target for LDL-C <1.8 mmol/L[1]. The ESC/EAS 2019 guideline recommends LDL-C reduction of 50% from baseline level (if statin naive), and an LDL-C <1.4 mmol/L[2]. However, LDL-C measurements are known to display (natural) variation over time within a patient. Therefore, the interpretation of an LDL-C measurement - in relation to the proposed treatment targets - is dependent on this variability. To investigate the within-patient variability of LDL-C in statin-treated post-ACS patients and how this variability may interfere with current guidelines adherence, we used the observational BIOMArCS study which employed high-frequency blood sampling in patients in the first year after ACS[3].

We performed a batchwise analysis of 1783 blood samples of 157 post-ACS patients (median of 11 per patient (range 3-19)) and measured total cholesterol, triglycerides and HDL-C levels using Beckman Coulter AU5811 (analytical variation <5%). LDL-C levels were calculated using the Friedewald formula. Included patients met the following conditions: statin use in same dose and sort confirmed at every sampling moment; free of recurrent cardiac events; ≥3 blood samples available taken 30 days after index-ACS; samples taken during the first 30 days were discarded. We calculated the within-patient coefficient of variation (CV_i) and the corresponding reference change value (RCV), which reflects the limit of (relative) change in biomarker values that can still be explained by combining within-patient and analytical variation.

Mean ± standard deviation (SD) age of the patients was 64 ± 8 years, 79.6% were men, and mean ± SD BMI was 27.4 ± 3.6. At the index-ACS, hypercholesterolemia was present in 51.0%, hypertension in 49.0%, and diabetes in 25.5%, whereas 47.7% and 1.9% were chronic statin and ezetimibe users.

Individual LDL-C distributions are depicted in Figure 4.1. Median (range) of patient's average LDL-C level was 2.23 mmol/L (1.08 – 3.28). The CV_i was 10.9%, with a corresponding RCV of 32.7%. The observed within-patient variability can potentially lead to clinically important differences in repeated samples. For example, in a subject with a habitual LDL-C of 1.8 mmol/L, measurements ranging between 1.2

and 2.4 mmol/L may well be explained by daily variation (figure). Obviously, these variations may then inappropriately reclassify patients above or below the treatment threshold, and, thus, will lead to over- or under- treatment.

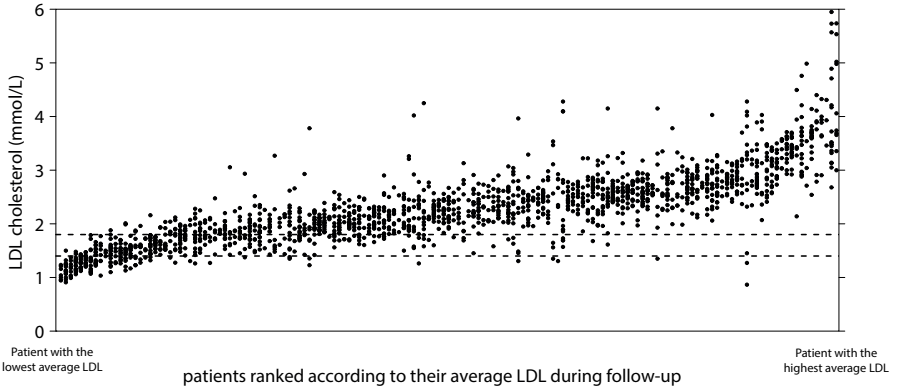

Figure 4.1 The X-axis represents the patients ranked on their average LDL-C value throughout the follow-up. Thus in the far left is the patient with the lowest average LDL-C throughout the year, while on the far right is the patient with the highest average LDL-C. The dots that are vertically aligned can thus be contributed to a single patient and represent all the measurements taken during the follow-up.

The use of LDL-C treatment targets have been questioned previously. Scientific evidence for current treatment targets is based on the observation that lower LDL-C levels are associated with lower cardiovascular risk[4], although all lipid lowering landmark trials to date have compared a therapy with a placebo or a standard of care[5-7]. Moreover, these trials included mostly white populations with an under presentation of other ethnic groups which impedes generalizability of the study results[8]. Finally, the set treatment targets cannot be met in most patients with (only) a statin[9, 10] (figure). This implicates that many post-ACS patients would need additional treatment, whilst neither adding ezetimibe nor adding PCSK9 inhibitor to statin treatment has shown to have effect on mortality[5-7]. Our results provide an additional argument against established treatment goals.

We used systematically, high-frequency blood sampling to accurately determine the LDL-C within-patient variability. Nevertheless, a few limitations can be

identified, such as using non-fasting samples, lacking data on compliance and not taking into account lifestyle or nutritional changes. However during regular outpatient visits of real-world post-ACS patients such variables are largely unknown as well. Given the observational nature of our study, with only regular contacts with the treating physician, we feel confident that the within-patient variability reported in our study represents the LDL-variability in daily clinical practice.

A final limitation is that the observed variability might have been influenced by the statin type and dosage. These were not recorded in BIOMArCS. However, to minimize this, we selected patients continuously treated with the same statin type and dosage throughout follow-up.

Conclusion

The within-patient variability of LDL-C in a real world setting in statin-treated high-risk patients can lead to clinically important discrepancies in subsequent LDL-C measurements. This variability impedes the correct assessment whether treatment goals have been met, and warrants much more critical appraisal in clinical practice than currently given.

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Part II

Prognostic value of repeated blood biomarker measurements in patients with acute coronary syndrome

Chapter 6

Temporal evolution of Myeloperoxidase and Galectin 3 during 1 year after acute coronary syndrome admission

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Abstract

Prior studies reported that Myeloperoxidase and Galectin-3, which are biomarkers of coronary plaque vulnerability, are elevated in acute coronary syndrome (ACS) patients. We studied the temporal evolution of these biomarkers early after ACS admission and prior to a recurrent ACS event during 1 year follow-up.

Introduction

Myeloperoxidase (MPO) and Galetin-3 (GAL-3) are pro-inflammatory proteins that promote plaque vulnerability through various mechanisms such as nitric oxide catalysation, foam cell formation and vascular smooth muscle cell dedifferentiation[1,2]. Both biomarkers, measured at admission, have been associated with cardiac death and non-fatal myocardial infarction (MI) during follow-up in patients with acute coronary syndrome (ACS)[1,3,4]. Since plaque vulnerability and thus coronary artery disease (CAD) is a highly dynamic process, repeated measurements of MPO and GAL-3 during follow-up may contain additional predictive value in post-ACS patients. To evaluate this hypothesis, we studied the evolution of these biomarkers in detail by means of highly frequent serial measurements during one year after ACS admission.

Methods

Study design

The BIOMarker study to identify the Acute risk of a Coronary Syndrome (BIOMArCS) was designed to reveal temporal evolutions of cardiovascular (CV) biomarkers during 1 year follow-up in ACS patients[5,6]. Differences in temporal changes between patients with and without a recurrent ACS (reACS) were of particular interest. A total of 844 patients were enrolled in 18 Dutch hospitals, who were aged ≥40 years and had ≥1 CV risk factor. Blood sampling was scheduled every two weeks during the first half-year and monthly during the second half-year, with the first sample taken at admission or at the first outpatient visit (4-6 weeks) after discharge. The study endpoint was defined as the composite of cardiac death, MI, or unstable angina requiring urgent coronary revascularization, and was reached by 45 patients. BIOMArCS was approved by the Institutional Review Boards of all participating hospitals, and all patients gave informed consent. BIOMArCS is registered in The Netherlands Trial Register as NTR1698.

Case-cohort approach

A case-cohort approach was used for biomarker determination and analysis of the temporal evolution during 1-year follow-up[7]. A case-cohort comprises a random

sub cohort from the full cohort, together with all patients who reach the study endpoint ('cases'). It is an efficient analysis method, while study validity and statistical power are maintained[8]. We selected a random sub cohort of 150 patients, which appeared to include 8 cases. Hence, our case-cohort consisted of (all) 45 study endpoint cases and 142 event-free patients. A median of 8 (interquartile range [IQR] 5-11) repeated samples were analyzed per patient, totalling 1478 measurements.

In order to obtain detailed information on biomarker changes early after ACS admission, by design, a series of 68 BIOMArCS patients underwent additional blood sampling at day 1 to 4. We included these patients in an analysis of post-ACS biomarker stabilization, excluding all 45 study endpoint cases to avoid distortion of the biomarkers patterns. As 19 (out of the 68) patients were also part of the case-cohort, a total of 191 patients contributed to a median of 8 (IQR 5-10) repeated samples per patient totalling 1507 measurements for this analysis[7].

MPO and Gal-3 measurements

Blood samples were collected on-site and frozen at -80℃ within 82 (25th-75th percentile 58-117) minutes after withdrawal. Subsequently, samples were securely transported to the Erasmus MC for long-term storage. Serum samples were used to measure MPO and GAL-3 and quantified batch-wise, blinded for patient characteristics. MPO was measured with a 384-ELISA plate (Nunc, Thermo #460372), with a lower limit of detection of 609 pg/ml. The corresponding 10% coefficient of variation was 5.7%. GAL-3 was measured with a custom built Luminex immune-assay validated in the University Medical Centre Utrecht, the Netherlands. The corresponding lower limit of quantification was 0.06 pg/ml, the upper limit of quantification was 1000 pg/ml and the reference sample value was 158.43 pg/ml. The inter-assay coefficient of variation of the used GAL-3 custom build assay was 13.9% and the intra-assay coefficient of variation was 14.45%.

Statistical analysis

MPO and GAL-3 had skewed distributions, and were log-transformed for analysis purposes. Results are presented on the linear scale.

Linear mixed-effect models (LME) were applied to describe the patterns of MPO and GAL-3 early after the index-ACS. We placed two splines to account for possible non-linearity. Using LME, we calculated the average biomarker values for each post-ACS day. We concluded biomarker stabilization when the (relative) difference in biomarker level between two consecutive days appeared less than one percent.

Joint models, combining LME and Cox proportional hazard regression models, were applied to study the temporal biomarker trajectories in relation to reACS[7]. We included time from index-ACS as main determinant, while adjusting for GRACE risk score, gender, history of diabetes, coronary artery bypass graft, valvular heart disease, and peripheral vessel disease. In the Cox model, GRACE risk score was added as potential confounder of the relation between biomarker level and the time-to-event. Additionally, we performed a post-hoc sensitivity analysis using only the data available after biomarker level stabilization to investigate if findings are influenced by early post-ACS elevations and variations in biomarker level.

Results of the joint models are presented as hazard ratios (HR) with corresponding 95% confidence interval (CI) per standard deviation (SD) increase of the biomarker (on the log-scale). All relevant model assumptions were evaluated, including residual plots, and no meaningful deviations were observed.

Analyses were performed with R Statistical Software using packages *nlme* and *JMbayes*. All statistical tests were two-tailed and the α-level of 0.05 was applied to conclude statistical significance.

Results

Median age was 63.6 (25th-75th percentile 55.3-71.6) years, 79.0% were men and index-ACS was classified as STEMI in 43.3% (Table 6.1). Cases had higher prevalence of diabetes and a higher GRACE risk score than event-free patients.

Myeloperoxidase

MPO level was elevated early after the index-ACS, with a peak value of 78.0 ng/ml at the day of admission. Within the first seven days, MPO showed a steep decline,

and then stabilized after day 6 at 25.7 ng/ml (Figure 6.1A). During follow-up, MPO levels in cases and event-free patients were similar: after seven days, the average serum level of MPO was 26.4 (IQR: 22.1-32.4) ng/ml in cases and 25.3 (IQR: 19.9-31.9) ng/ml in endpoint-free patients. We did not observe a steady or sudden increase in MPO level prior to the reACS event (Figure 6.1B). The unadjusted HR for reACS per SD increase in MPO was 0.84 (95% CI 0.61-1.26) Adjustment for multiple factors did not result in a meaningful change of the estimate (Table 6.2).

Table 6.1 Baseline characteristics.

	Endpoint cases	Endpoint-free patients	p-value
Number of patients	45	142	
Age, yr (IQR)	67.4 (57.1-76.5)	62.6 (55.0-70.9)	0.075
Man (%)	36 (80.0)	111 (78.2)	0.79
Cardiovascular risk factors (%)			
Diabetes Mellitus	17 (37.8)	24 (16.9)	0.003
Hypertension	22 (48.9)	77 (54.2)	0.53
Hypercholesterolemia	20 (44.4)	72 (50.7)	0.46
Current smoker	17 (37.8)	60 (42.2)	0.52
Presentation on admission			
Diagnosis			
STEMI	16 (35.6)	65 (45.8)	0.46
NSTEMI	22 (48.9)	56 (39.4)	
Unstable angina pectoris	7 (15.6)	21 (14.8)	
PCI performed	34 (87.2)	109 (82.6)	0.50
GRACE risk score (IQR)	121 (98-141)	109 (88-130)	0.022

Continuous variables are presented as median with IQR. Categorical variables are presented as numbers and percentages. GRACE risk score: Global Registry of Acute Coronary Events risk score, NSTEMI: non-STEMI, PCI: Percutaneous coronary intervention, STEMI: ST-elevation myocardial infarction, yr: year.

Galectin-3

Gal-3 was only slightly elevated at the index-ACS, and stabilized after day 3 at 0.21 ng/ml (Figure 6.1C). Gal-3 remained constant during follow-up, and mean levels did not differ between cases and event-free patients (Figure 6.1D). After 7 days, the average serum level of GAL-3 was 0.24 (IQR: 0.16–0.30) ng/ml in cases and 0.23 (IQR: 0.17–0.30) ng/ml in endpoint-free patients. Prior to reACS, we observed no steady or sudden elevation in GAL-3 in cases. The unadjusted HR for reACS per SD increase in GAL-3 was 1.41 (95% CI 0.77-2.42), which remained unaltered after multiple adjustment (Table 6.2).

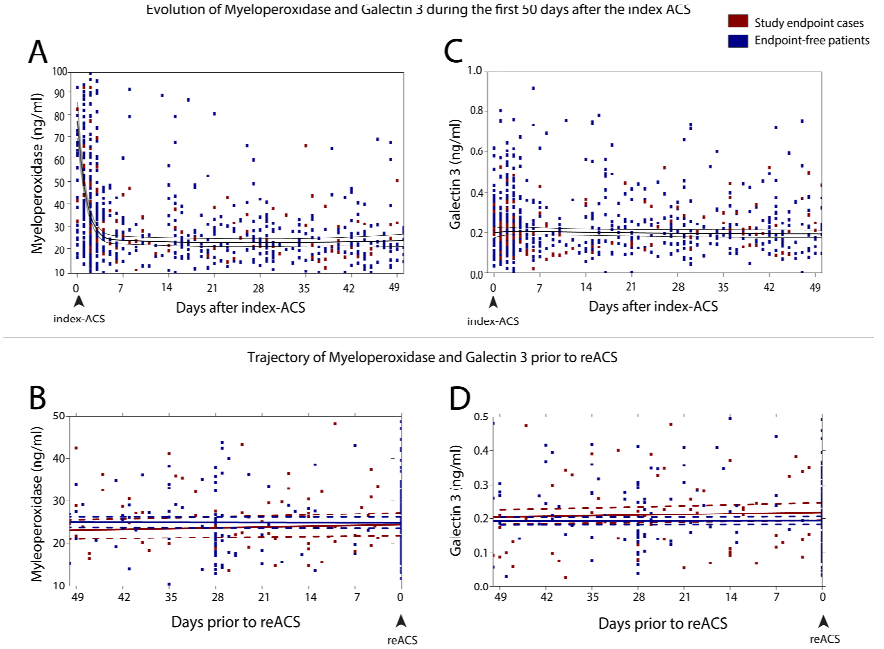

Figure 6.1 Temporal evolution of Myeloperoxidase and Galectin 3. Panel 1A and 1C depict the early time-course of MPO and GAL-3 after the index-ACS. Panel 1B and 1D depict the median value of the patient-level mean of MPO and GAL-3 prior to reACS in study endpoint cases and endpoint-free patients.

Table 6.2 Associations between biomarker trajectories and study endpoints.

	- 1 SD	Mean	+ 1 SD	unadjusted HR	p-value	Adjusted HR*	p-value
Myeloperoxidase, ng/ml†	15.6	26.2	44.0	0.84 (0.61-1.26)	0.32	0.84 (0.56-1. 37)	0.44
Galectin 3, ng/ml†	0.10	0.20	0.38	1.41 (0.77-2.42)	0.27	1.56 (0.87-2.53)	0.12
Sensitivity analysis with post 7 days data							
	- 1 SD	Mean	+ 1 SD	unadjusted HR	p-value	Adjusted HR*	p-value
Myeloperoxidase, ng/ml‡	15.7	24.7	38.8	1.00 (0.62-1.69)	1.00	1.18 (0.74–1.91)	0.52
Galectin 3, ng/ml‡	0.10	0.20	0.38	1.33 (0.63-3.09)	0.47	1.02 (0.48-2.15)	0.95

† based on 1478 measurements in 187 patients (median: 8 [IQR: 5-11]). ‡ based on 1282 measurements in 174 patients (median: 8 [IQR: 4-10]). *Cox model adjusted for GRACE risk score, mixed model adjusted for GRACE risk score; gender; history of diabetes, coronary artery bypass graft, valvular heart disease, peripheral vessel disease. HR: hazard ratio; ml: microliter; ng: nanogram; SD: standard deviation.

Discussion

We established the detailed temporal trajectories of MPO and GAL-3 in post-ACS patients by means of frequently serial measurements. MPO was elevated at the time of the index-ACS, and decreased and stabilized within 7 days. Longitudinal MPO levels were not associated with reACS. In particular, no increase in MPO level was observed prior to a recurrent event. Similar results were observed with respect to GAL-3: there were no differences in longitudinal evolution between reACS cases and event-free patients.

MPO is a pro-inflammatory biomarker involved in multiple inflammatory processes that propagate plaque instability, such as nitric oxide catalysation, leukocyte attraction, endothelial cell apoptosis and tissue factor activation stimulating thrombosis[2]. GAL-3 is also reckoned a pro-inflammatory biomarker stimulating plaque instability by i.e. monocyte attraction, macrophage polarization, foam cell production and vascular smooth muscle cell dedifferentiation[1]. Because of their inflammatory character, MPO and GAL-3 may destabilize plaques susceptible to thrombosis, leading to reACS in post-ACS patients[1,9]. A recent meta-analysis showed that higher MPO levels measured at baseline, are associated with adverse outcome[9]. As for GAL-3, opposite results have been found regarding its prognostic value in post-ACS patients[10-12].

BIOMArCS was specifically designed to study the temporal evolution of serum biomarkers in post-ACS patients, and its highly frequent blood sampling schedule would have sufficed to identify meaningful changes in MPO and GAL-3 concentrations, had they appeared. However, contrary to our expectations, both biomarkers were not associated with an increased risk of a recurrent ischemic event during 1-year follow-up. Since the median time between the last collected sample in cases and their reACS was 11 (IQR: 5-20) days, we cannot exclude that just before reACS there might still have been biomarker elevations we did not detect. Additionally, we cannot exclude changes in MPO or GAL-3 levels during long-term follow-up.

Nonetheless, it seems that MPO and GAL-3 do not advance plaque vulnerability prior to reACS.

Conclusion

MPO and to a lesser extend GAL-3 were elevated early after, but not before a clinical symptomatic ACS. Post-ACS patients who experienced a recurrent event within one year were not characterized by elevated levels of these pro-inflammatory biomarkers. Also steady or sudden elevations were absent, hence MPO and GAL-3 appear unsuited for prognosis monitoring after ACS.

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Chapter 7

Evolution of renal function and predictive value of serial renal assessments among patients with acute coronary syndrome: BIOMArCS study

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Abstract

Background

Renal dysfunction predicts mortality in acute coronary syndrome (ACS), but its evolution following and preceding ACS has never been described in detail. We aimed to describe this evolution using serial measurements of creatinine, glomerular filtration rate [eGFR], and cystatin C [CysC].

Methods

From 844 ACS patients included in the BIOMArCS study, we analysed patient-specific longitudinal marker trajectories from the case-cohort of 187 patients to determine the risk of the endpoint (cardiovascular death or hospitalization for recurrent non-fatal ACS) during 1-year follow-up. Study included only patients with eGFRCr≥30 ml/min/1.73 m^2. Survival analyses were adjusted for GRACE risk score and based on data <30 days after the index ACS (mean of 8 sample per patient).

Results

Mean age was 63 years, 79% were men, 43% had STEMI, and 67% were in eGFR stages 2–3. During hospitalization for index ACS (median [IQR] duration: 5 (3–7) days), CysC levels indicated deterioration of renal function earlier than creatinine did (CysC peaked on day 3, versus day 6 for creatinine), and both stabilized after two weeks. Higher CysC levels, but not creatinine, predicted the endpoint independently of the GRACE score within the first year after index ACS (adjusted HR [95% CI] per 1SD increase: 1.68 [1.03–2.74]).

Conclusions

Immediately following index ACS, plasma CysC levels deteriorate earlier than creatinine-based indices do, but neither marker stabilizes during hospitalization but on average two weeks after ACS. Serially measured CysC levels predict mortality or recurrence of ACS during 1-year follow-up independently of patients' GRACE risk score.

Introduction

Renal dysfunction, including mild renal impairment (eGFR 60-89 ml/min/1.73 m^2)[1,2], is strongly associated both with short- and long-term mortality in patients with ST elevation myocardial infarction (STEMI) and in those with non-STEMI[3-5]. Patients with chronic kidney disease (CKD) are often treated less aggressively for acute coronary syndrome (ACS) than those without CKD[3,4,6]. However, even if they are on optimal therapy they will still have poorer prognosis[7]. Renal dysfunction is associated both with coronary atherosclerosis (e.g., higher coronary plaque burden, plaques containing greater necrotic core and more dense calcium), and with abnormalities of cardiac muscle (e.g., left ventricular hypertrophy, dilated cardiomyopathy, and systolic dysfunction)[8-10]. Several studies have shown that specific comorbidities such as hypertension, diabetes, and dyslipidemia, contribute both to cardiovascular and renal damage[11,12]. Neuro-hormonal activation is also affected after ACS[13-15],http://eurheartj.oxfordjournals.org/content/24/5/412.long -ref-20 and angiotensin II may influence deterioration of both cardiovascular and renal functioning[13,16,17].

In heart failure (HF), renal dysfunction has been identified as the most prevalent comorbidity and strongly predicted adverse clinical out-comes[18,19]. Worsening renal function has also been used as the primary endpoint in several clinical trials in acute HF[20,21]. Underlying hemodynamic dependence between the heart and kidneys including renal perfusion hemodynamics and systemic neuro-hormonal activation, has been identified as the main driver of such a relationship[22].

In spite of these overlapping pathophysiological aspects, the detailed temporal evolution of renal function immediately following ACS, and during the year preceding recurrent ACS, has not yet been described. Existing studies have mostly assessed renal function only at a single time point to investigate prognostic value, and have used for example time of admission, a moment during in-hospital stay or time of discharge as 'study baseline'. However, it is unclear whether a patient's renal function examined at these time points during hospitalization reflects "true" renal functioning or whether it may be temporarily disturbed by the index ACS. Moreover, it remains unknown at which moment after ACS renal function stabilizes. Knowing these temporal patterns may help us in expanding our understanding of renal dysfunction in patients with ACS, and thereby aid in identifying high-risk subgroups.

The aim of our study was two-fold: (1) to describe the evolution of renal function from its initial change during ACS until stabilization, according to the kinetics of several renal function parameters (creatinine, estimated glomerular filtration rate [eGFR], and cystatin C [CysC]), (2) to investigate the predictive value of serial renal assessments within the first year after index ACS in patients with normal to moderately-reduced renal function. For the latter purpose, we also examined whether rates of change of these renal markers are relevant for clinical risk prediction.

Methods

BIOMArCS

BIOMArCS is a multi-centre prospective study conducted in 18 Dutch hospitals[23]. Details on the BIOMArCS design are reported elsewhere[24]. Briefly, we included patients who were hospitalized for ACS including STEMI, non-STEMI, and unstable angina pectoris(UAP), with ≥1 cardiovascular risk factor (Table S7.1); eGFR<30 ml/min/1.73 m^2 was an exclusion criterion because of the potential influence of renal clearance on certain biomarkers investigated in the BIOMArCS cohort[23]. All patients were treated according to prevailing guidelines and at the discretion of the treating physician. The study protocol has been approved by the Institutional Review Board of all participating hospitals and written informed consent was obtained from all patients

Selection of patients to analyse the relation between renal markers and repeat ACS

For the analysis of the relation between (renal) biomarkers and repeat ACS during 1-year follow-up, we applied a case-cohort design, which allowed a comparison of all study endpoint cases to a limited random sample of non-cases (instead of all non-cases), thereby increasing the study's efficiency[25]. For this purpose, after study completion (i.e., inclusion, follow-up, and study endpoint adjudication) a sub-cohort of 150 patients was randomly sampled from the parent cohort (n=844), using a computer generated random sampling procedure. Subsequently, all patients who experienced the endpoint, but who were not a part of the random sub-cohort were added (37 cases), so that the case-cohort comprised 187 patients

(Figure 7.1). Thus, we analysed all cases, but analysed only those non-cases (non-endpoint patients) who were present in the random sub-cohort.

Selection of patients to analyse the washout of renal markers immediately following index ACS

To enable a precise description of early washout biomarker patterns, a total of 68 (8%) BIOMArCS patients underwent additional blood sampling at 24, 48, 72 and 96 h after the index ACS. We excluded the 6 patients who experienced the study endpoint within the first two month due to potential influence on stabilization of the washout pattern, and enriched with the endpoint-free patients from the random sub-cohort. Thus, a total of 185 patients were available for the analysis of washout patterns of renal biomarkers (Figure 7.1).

Follow-up visits and blood sample collection

Blood samples were collected at admission, hospital discharge, and every two weeks after index ACS during the first six months, followed by monthly collection until one year (Figure 7.1). A visit window of ±1week was allowed, and a maximum of two consecutive visits were allowed to be skipped (for personal reasons). If logistic reasons hindered inclusion during hospitalization, patients could be included on the first outpatient visit within six weeks after discharge; the sampling schedule was then adapted accordingly. A trained research nurse interviewed the patients at each visit and obtained data on anginal status(Canadian Cardiovascular Society classification), HF symptomatology (New York Heart Association classification), and factors that might influence biomarker levels, e.g. smoking, occurrence of infections, inflammatory or allergic responses, alterations in medication, interventional or operative procedures and hospital admission.

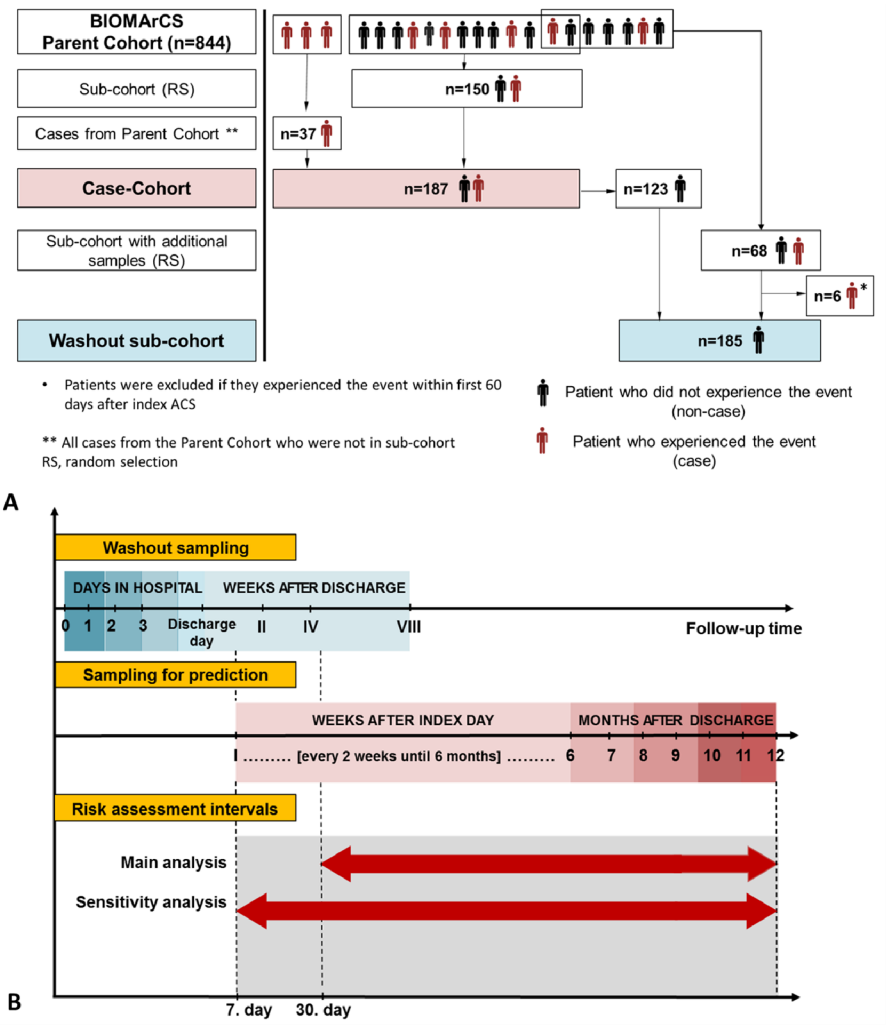

Figure 7.1 Participants flow chart, study design, and sampling schema. Case-Cohort was constructed from a random sample of 150 patients from the full cohort (n=844, all enrolled patients) and enriched with all cases (n=37). For the case-cohort, blood samples were collected at admission, at hospital discharge, and subsequently every two weeks during the first six months, followed by monthly collection until 1 year(sampling for prediction). Risk assessment time intervals were:(1) Main analysis >30 days until study endpoint or last sample moment,(2) Sensitivity analysis >7 days until study endpoint or last sample moment. Washout sub-cohort was constructed from a random sample of 68 patients from the parent

cohort in whom additional samples were collected within 24, 48, 72 and 96 h after admission, at the day of hospital discharge, and at 2, 4 and 8 weeks (washout sampling). Patients who experienced new events within the first 60 days form the index ACS were excluded due to potential influence on stabilization of the washout pattern (n=6). The washout sample was then enriched with123 patients who did not experience incident events from the sub-cohort of 150 random patients, resulting in a total of 185 patients for the washout sub-cohort.

Blood samples were processed on-site and transported batch-wise under controlled conditions to the department of Clinical Chemistry of the Erasmus MC, Rotterdam where they were stored until analysis was performed.
Glomerular filtration rate (GFR) was determined by the Modification of Diet in Renal Disease (MDRD) Study equation[26]. Patients were categorized using the modified eGFR definition from the National Kidney Foundation–Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines[27].

Analysis of renal markers

In the 187 case-cohort patients and in the 185 patients that comprise the washout analysis set, renal biomarkers (creatinine and CysC) were measured batch-wise at the laboratory of the department of Clinical Chemistry and Hematology of the University Medical Center Utrecht. Creatinine was measured on clinical routine equipment (AU5800, Beckman Coulter, Brea, CA, USA). Cystatin C was measured by ELISA following manufacturer's instructions (mouse-anti human DuoSet DY1196, R&D Systems, Oxon, UK; inter- and intra-assay CV<10%).The EDTA-plasma was used for biomarker analysis. Importantly, laboratory personnel were blinded to any patient data and scope of the study, whereas biomarker measurements did not interfere with treatment.

Study endpoints

The study endpoint was a composite of cardiac mortality or a diagnosis of a non-fatal myocardial infarction or unplanned coronary revascularization due to progressive angina pectoris during 1-year follow-up. Any death was considered cardiac unless documented otherwise. Incident non-fatal myocardial infarction was defined as the combination of typical ischemic chest complaints and objective evidence of myocardial ischemia or myocardial necrosis as demonstrated by the ECG and/or elevated cardiac markers. The criteria for non-fatal myocardial

infarction during follow-up were the same as those for the index event (Supplementary Table S7.1, points 1 and 2 of the inclusion criteria). A Clinical Event Committee, blinded for the renal biomarker results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

Statistical analysis

Case-Cohort – prediction of events

Categorical baseline data are summarized by percentages, and continuous data by medians and 25^{th}-75^{th} percentiles. Differences between cases and non-cases were evaluated by classical statistical tests, as specified in the caption of Table 7.1.
To obtain valid inferences for the relation between the temporal evolvement of a biomarker and the incidence of the study endpoint, the longitudinal- and event-processes must be jointly modelled [28]. We applied Bayesian semiparametric joint models for this purpose, which combine linear regression and Cox proportional hazard regression. Linear mixed-effects (LME) models were used to describe patient-specific longitudinal biomarker trajectories B(t) as a function of time (t). Non-linear trajectories were modelled by cubic splines. ^{2}Log-transformations of biomarker values were used to assure normal distributions of regression residuals. More specifically, the unit of analysis was the Z-score (i.e. the standardized form) of the ^{2}log-biomarker, which allows a direct comparison of the effects of separate markers. Results are presented as hazard ratios (HR) and corresponding 95% confidence intervals (CI) for a 1 SD difference of the biomarker on the log-scale.

Table 7.1 Baseline characteristics of the parent cohort and case-cohort set.

Characteristics	All patients	Case-cohort		
		Non-cases	Cases	p-value
Number of patients	844	142	45	
Presentation and initial treatment				
Age, years, median (IQR)	62.5 (54.3, 70.2)	62.6 (55.0-70.9)	67.4 (57.1-76.5)	0.07
Male sex, %	77.9	78.2	80.0	0.79
Admission diagnosis, %				0.46
STEMI	51.7	45.8	35.6	
NSTEMI	37.7	39.4	48.9	
UAP	10.6	14.8	15.6	
Culprit artery, %				
RCA	33.1	34.5	26.7	0.33
LM	2.5	3.5	2.2	1.00
LAD	31.9	33.8	31.1	0.74
LCX	16.5	12.0	20.0	0.17

Table 7.1 (continued)

Characteristics	All patients	Case-cohort		
		Non-cases	Cases	p-value
CAG performed, %	94.4	93.7	89.0	0.33
PCI performed , %	86.3	82.6	87.2	0.49
$CK_{max,}$ U/L median (IQR)	513 (200, 1370)	449 (190, 1197)	389 (194, 1122)	0.78
Killip class, %				0.012
Class I		94	82	
Class II		4	16	
Class III		2	0	
Class IV		0	2	
Renal function on admission:				
Urea, mmol/L median (IQR)		5.9 (5.0-7.0)	6.8 (4.7-7.9)	0.19
Creatinine, umol/L median (IQR)		82 (69-95)	87 (73-93)	0.22
eGFR, mL/min/1.73 m^2 median (IQR)		83 (69-98)	78 (71-92)	0.21
KDOQI classification[a], (%)				
eGFR ≥90 mL/min/1.73 m^2		35	24	0.16
eGFR 60-89 mL/min/1.73 m^2		55	60	
eGFR 30-59 mL/min/1.73 m^2		10	16	
Medical history, %				
Diabetes mellitus	23.5	16.9	37.8	0.003
Hypertension	55.5	54.2	48.9	0.53
Dyslipidemia	49.3	50.7	44.4	0.46
Prior PCI	26.2	27.0	31.1	0.59
Prior CABG	10.0	8.5	24.4	0.004
Prior MI	26.9	30.3	31.1	0.92
Heart failure	2.4	2.8	8.9	0.097
Valvular heart disease	2.2	1.4	8.9	0.031
Prior CVA/TIA	9.0	11.3	20.0	0.13
PAD	8.9	6.3	22.2	0.004
Medication at first blood sampling moment from7 day after index ACS, %				
Aspirin	95.1	93.0	100	0.20
P2Y12 inhibitor	94.8	90.4	96.8	0.46
Vitamin K antagonist	6.9	7.9	9.7	0.72
Statins	95.8	95.6	96.8	1.00
Beta-blocker	90.1	85.1	93.5	0.37
ACE inhibitor or ARB	83.6	84.2	90.3	0.57

ACE: angiotensin converting enzyme; ARB: angiotensin II receptor blocker; CABG: coronary artery bypass grafting; CK_{max}: maximum creatine kinase during the index admission; LAD: left anterior descending artery; LCX: left circumflex artery; LM: left main coronary artery; NSTEMI: non-ST-elevation myocardial infarction; PCI: percutaneous coronary intervention; RCA: right coronary artery; STEMI: ST-elevation myocardial infarction; SD: standard deviation; $Troponin_{max}$: maximum troponin value during the index admission; UAP: unstable angina pectoris.

The LME models not only provide unbiased estimates B(t) of the biomarker level at timepoint t, but also of its instantaneous rate of change (or: slope) B'(t) at t, that corresponds to the first derivative of B(t). Since we also aimed to study rate of

change, we also provided HRs for the instantaneous slope of the marker's trajectory. Further details on this method of dynamic prediction modeling were described elsewhere[29]. Results are presented as HRs (95% CIs) for a 0.1 SD difference of the marker's rate of change on the log-scale.

Analyses were first performed univariably, and subsequently multivariable adjustment was performed. For this purpose, the GRACE risk score for assessment of post-discharge death and myocardial infraction, as recommended by international guidelines[30-32], was used. This specific GRACE risk model consists of age, troponin (or CKMB) elevation at admission, history of MI, congestive heart failure and whether CABG was performed at the index hospitalization[33]. The survival model was adjusted for the GRACE risk score, and the LME model was adjusted for GRACE risk score, sex, diabetes, history of coronary artery bypass surgery, history of valvular heart disease, history of stroke, history of peripheral arterial disease.

To describe the average evolution of renal function during the year preceding death or the recurrence of ACS, we analyzed all available data >7 days after the index ACS until the endpoint or last sample moment.

To investigate the predictive value of repeatedly measured markers, we analysed all available data >30 days after the index ACS event, to ensure that all biomarkers were then stabilized. Additionally, a sensitivity analysis was performed on all repeated measurements >7 days after the index ACS. Measurements that were obtained within 7 days after index ACS were excluded to avoid biased estimates due to elevated biomarkers induced by the index ACS.

Analysis of evolution of renal function during the washout phase

LME models were applied to investigate at which time point the renal markers reach their highest point (creatinine, CysC) or lowest point (eGFR) and at which time point they return to stable levels. All renal biomarkers were ^{2}log transformed, and non-linear evolutions (for the fixed- and random-effects parts) were modelled by restricted cubic splines. We optimized the position of the spline knots by using Akaike information criteria (AIC) and Bayesian Information criteria (BIC). After obtaining optimal evolution curves representing the washout patterns of the renal markers, we calculated the maximum or minimum of these curves to determine the time point of the peak or nadir. To determine the moment of marker stabilization, we also numerically compared the deltas of biomarkers between every two consecutive blood samples (a difference <1% signified a stabilization).

R statistical software (version 2.15.0) was used for advanced statistical analyses, in particular the package JMbayes[28]. All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Results

Study endpoints and baseline characteristics

Of 844 enrolled patients, 45 reached the study endpoint during a median (IQR) follow-up of 11.5 (2.7–12.1) months. Baseline characteristics of all patients in the BIOMArCS study and in the case-cohort set are shown in Table 7.1. In the case-cohort, on admission mean (±SD)age was63 (±11) years, 79% were men, 43% had STEMI, 42% had non-STEMI, and 15% had UAP. The median (IQR) eGFR was 81 (70-98) ml/min/1.73 m^2, and 33% of patients were in eGFR stage 1 (GFR≥90), 56% in stage 2 (GFR 60–89), and 11% in stage 3 (GFR 30–59).

Average evolution of renal markers immediately following index ACS

A total of 687 samples were drawn from the 185 non-endpoint patients that comprise the washout analysis set, with a mean of 4 samples per patient. Average washout evolutions of plasma creatinine, eGFR and CysC are shown in left panel of Figure 7.2. The figure shows that CysC levels reached a peak on the 3rd day after index ACS. This was followed by a nadir of eGFR on the 4th day, and a peak of creatinine levels on the 6th day. We also found different time intervals from the highest or lowest point to stabilization for these markers: CysC–11 days (stabilized on day 13), eGFR–10 days (stabilized on day 13) and creatinine–8 days (stabilized on day 14). Nevertheless, the stabilization of the markers after index ACS appeared to be temporary.

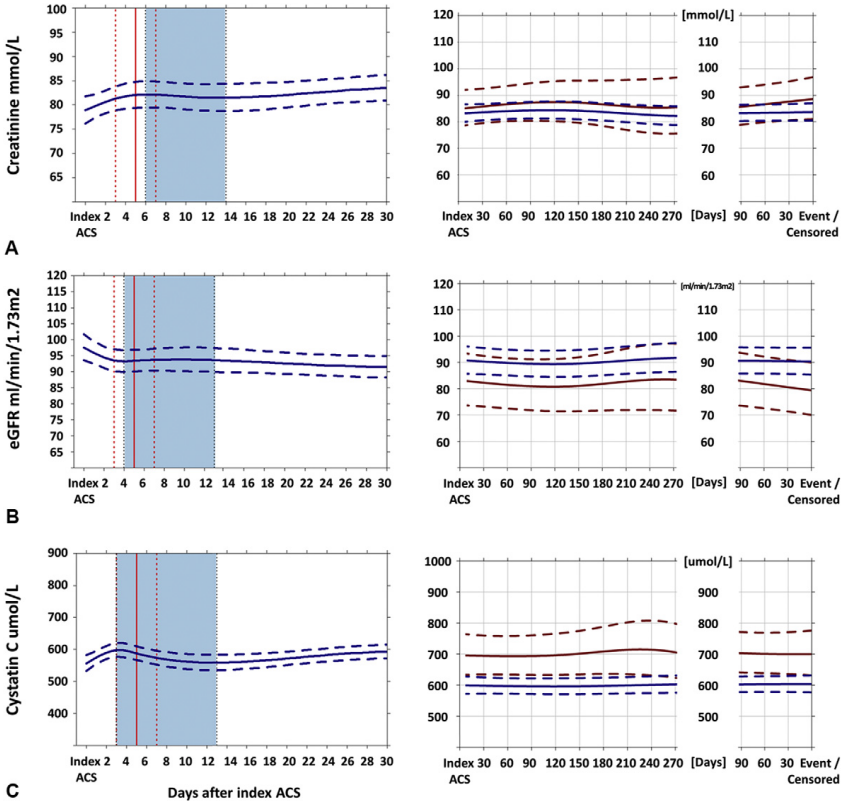

Figure 7.2 Average evolution of renal markers immediately following index ACS and during the year preceding death or recurrence of ACS or last sample moment. Legend: Left panel: the follow-up time (days) starting from admission is displayed on the x-axis. Renal marker levels are displayed on the y-axis. The solid red line depicts the median discharge day from hospital with corresponding interquartile range (dashed red lines). The left black dashed line displays time of the highest peak of plasma creatinine and cystatin C and the lowest peak of eGFR, and the right black dashed line displays the time moments of biomarker stabilization. The light blue area (between the two black dashed lines)represents the time period from the peaks/nadirs to stabilization. Right panel: the solid red line depicts the average evolutions of renal markers in patients who reached the endpoint, and the solid blue line depicts the evolutions in endpoint-free patients. The dashed lines represent the 95% confidence interval. A. plasma creatinine (mmol/L); B. eGFR (ml/min/1.73 m^2); C. plasma cystatinC (µg/ml).

Average evolution of renal markers during the year preceding death or next ACS

In the time-period >30 days after index ACS, a total of 1117 blood samples were collected from 158 of the 185 patients that comprise the case-cohort, with a median of 7 samples per patient - the remaining 27 patients (17 study endpoint cases) only had samples in the 0–30 day time window. Although plasma creatinine levels increased slightly prior to the incident event in patients who ultimately reached the study endpoint, substantial overlap was present between average evolutions of these patients and those who remained endpoint-free (Figure 7.2: right panel). eGFR displayed similar dynamics, but with a smaller overlap. Notably, plasma CysC showed substantially higher levels during follow-up in patients ultimately reaching the study endpoint.

Predictive value of renal markers during the year preceding death or next ACS

Higher levels of CysC assessed at any point in time during follow-up were positively associated with the endpoint (HR [95% CI]: per 1SD increase of 2logCysC: 1.79 [1.21–2.63], p=0.006). After controlling for the GRACE risk score, CysC level remained a significant predictor (adjusted HR [95% CI]: 1.63 [1.01–2.66], p=0.043).In the sensitivity analysis, CysC level measured serially >7 days after the index ACS was slightly weaker, but also a significant predictor (1.68[1.13–2.46], p=0.009). After adjustment for the GRACE risk score, the risk estimates remained materially the same (adjusted HR [95% CI]:1.63 [1.01–2.57], p=0.045) (Table S7.2).
No clear associations were found between serially assessed plasma creatinine or eGFR and the study endpoints (Table 7.2).
None of the slopes of the renal markers trajectories were associated with the endpoint (Table 7.2, and Table S7.2).

Table 7.2 Hazard ratios for the primary endpoint in relation to serially assessed marker levels >30 days after index ACS.

	Geometric mean**			Levels[a]		Instantaneous Slope[b]	
	Mean - 1 SD	Mean	Mean + 1 SD	HR (95% CI)	p-value	HR (95% CI)	p-value
Creatinine	67	84	105				
crude model				1.28 (0.84-1.97)	0.28	1.00 (0.53-1.85)	0.98
+ GRACE risk score[#,*]				1.12 (0.73-1.76)	0.61	1.00 (0.53-1.89)	0.99
eGFR	64	88	120				
crude model				1.52 (0.97-2.37)	0.06	1.00 (0.53-1.86)	1.00
+ GRACE risk score[#,*]				1.32 (0.85-2.10)	0.20	1.02 (0.56-1.87)	0.93
CysC	473.1	613.1	794.6				
crude model				1.79 (1.21-2.63)	0.006	0.99 (0.53-1.90)	0.98
+ GRACE risk score[#,*]				1.63 (1.01-2.66)	0.043	0.99 (0.53-1.83)	0.99

[a]Hazard ratios (HRs) and 95% confidence interval (CI) are given per 1-SD increase (creatinine and cystatin C), and 1-SD decrease (eGFR) on the 2-log scale at any time point after 30 days after index ACS. [b] HRs (95%) CI are given per 0.1-SD increase in the slope (creatinine and cystatin C), and 0.1-SD decrease (eGFR) on the 2-log scale at any time point after 30 days after index ACS. [#] longitudinal model adjusted for GRACE risk score, sex, diabetes, history of coronary artery bypass surgery, history of valvular heart disease, history of stroke, history of peripheral arterial disease. [*] survival model adjusted for GRACE risk score. ** Geometric mean ± 1 standard deviation (SD) of the patient-specific biomarker values after 30 days (presented on the linear scale).

Discussion

In this prospective multicenter study, we sought to describe the longitudinal trajectories of different renal markers, and their impact on 1-year cardiac outcome in patients with ACS. We found that plasma CysC levels predict mortality or recurrence of ACS within the first year independently of patients' GRACE risk score. We also found that CysC levels deteriorate earlier than creatinine-based indices do during index ACS. Importantly, we observed that both renal markers usually do not stabilize during hospitalization, but on average two weeks after index ACS. Altogether, these findings underscore the relation of renal dynamics with ACS, and carry implications for the monitoring of renal function in these patients.

The majority of studies in patients with ACS have focused on prognostic value of creatinine levels or estimated GFR assessed at one point in time. However, the prognostic value of serial renal assessments, including CysC levels, is less clear and has mainly been investigated in patients with HF[18,34]. In acute HF, studies have shown that worsening renal function during hospitalization entails poor prognosis especially if a patient's clinical status deteriorates simultaneously[35]. Otherwise,

small to moderate renal function decline during hospitalization in the setting of aggressive diuresis may simply be result of decongestion and clinically benign[36,37]. In chronic HF, serial measurements of creatinine and CysC during outpatient follow-up strongly predicted long-term adverse clinical outcomes such as HF rehospitalization and death[34].

In patients with ACS, some authors[38] have speculated that assessment of renal function should be repeated after hospital discharge to ensure that 'true' renal functioning is detected, and not transient renal fluctuations. However, no study has examined the evolution of renal function during the washout phase early after ACS and during 1-year follow-up. It is here that our study further extends existing evidence. Our findings suggest incremental value of CysC levels for risk assessment by means of the GRACE score. These findings are also supported by Correa et al.[39], who found that CysC levels predicted cardiovascular death or HF hospitalization in patients after ACS, independently of established cardiovascular risk predictors including troponins and brain natriuretic peptide. Interestingly, Correa et al. collected samples at a median of 14 days after ACS. This underpins findings from our washout cohort, indicating that CysC level usually stabilizes on average two weeks after ACS. Taken together, it seems reasonable to re-assess CysC levels in the time period after hospital discharge in patients for whom a more complete risk assessment is required.

Previous studies that also used repeated CysC measurements are scarce. Akerblom et al. assessed whether repeatedly measured CysC levels (at baseline, discharge, and the mean value of both measurements) carry predictive value in 4295 patients with ACS and similar baseline creatinine levels as those in our study[40]. They reported that serial CysC assessment did not improve risk prediction. However, our results were obtained using a different approach. Contrary to Akerblom et al., we examined long-term temporal evolution of renal markers, specifically by using repeated measurements up to 1 year after hospital discharge to estimate the CysC trajectories in each patient. We then jointly modelled these renal trajectories with time-to-event analysis. This joint modeling approach carries several advantages. It enabled us to investigate the association with adverse events in a less biased way[41]. It also allowed us to examine the associations between the rates of change of different renal function parameters and adverse events. The latter analyses suggested that although CysC levels contribute to a patient's clinical risk, their rates of change do not. This is supported by Shlipak et al. who also could not demonstrate a significant association between change in creatinine (delta-

creatinine ≥0.3 mg/dl) and outcomes in patients with stable coronary artery disease (CAD) in the Heart and Estrogen/Progestin Replacement Study (HERS)[42]. Thus, it appears that rate of change of renal function is only relevant for clinical risk in patients with CAD and systolic dysfunction, or with HF[18,34,38].

Although we observed a slight deterioration of creatinine-based estimates prior to the incident endpoint, we could not confirm their predictive value as found previously[1,2]. This may be explained by the relatively low prevalence of patients with more severe renal dysfunction in our study. In fact, only 11% of our patients had moderate renal impairment(eGFRC 59-30 ml/min/1.73 m^2) and there were no patients with eGFR <30 ml/min/1.73 m^2 due to the exclusion criteria. However, it appears that CysC levels were still able to detect these subtle differences, which may be of particular interest for patients with mild eGFR reduction (eGFRC 60-89 ml/min/1.73 m^2), as was the case in 56% of patients included in the study. Indeed, studies have shown that CysC levels correlate more closely with the true GFR than serum creatinine levels[43-45]. Although a possible non-renal link between CysC and cardiovascular risk has been suggested[46], a recent Mendelian randomization study by Van der Laan et al. could not substantiate a causal role of CysC in etiology of cardiovascular disease[47]. Finally, although such mild renal dysfunction usually does not require specific management, accurate monitoring of these subtle differences by CysC may carry potential for improving risk stratification of these patients.

Study limitations

Several aspects of our study warrant consideration. First, the MDRD equation, although validated in patients with ACS, has limitations due to the non-renal factors that influence creatinine measures. Likewise, proteinuria was not measured in this cohort. Nevertheless, we chose MDRD because it is the most widely utilized eGFR equation, and thus enables comparisons with existing studies. Second, patients were excluded in case of eGFR <30 ml/min/1.73 m^2, which limits generalizability of our results to the ACS population at large. Yet we were able to demonstrate, even in this ACS population with a lesser degree of renal impairment, that renal dysfunction quantified by plasma CysC is associated with cardiovascular events. Third, despite controlling analyses for GRACE risk score, a risk model recommended in international guidelines, residual confounding may still be present.

Conclusion

Immediately following index ACS, plasma CysC levels deteriorate earlier than creatinine-based indices do, but neither marker stabilizes during hospitalization but on average two weeks after ACS. Serially measured CysC levels predict mortality or recurrence of ACS within the first year independently of GRACE risk score.

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Chapter 8

High-frequency metabolite profiling and the incidence of recurrent cardiac events in post-acute coronary syndrome patients

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Biomarkers. 2020

Abstract

Purpose

The aim of this study was to study temporal changes in metabolite profiles in patients with post-acute coronary syndrome (ACS), in particular prior to the development of recurrent ACS (reACS).

Methods

BIOMArCS (BIOMarker study to identify the Acute risk of a Coronary Syndrome) is a prospective study including patients admitted for ACS, who underwent high-frequency blood sampling during 1-year follow-up. Within BIOMArCS, we performed a nested case-cohort analysis of 158 patients (28 cases of reACS). We determined 151 metabolites by nuclear magnetic resonance in seven (median) blood samples per patient. Temporal evolution of the metabolites and their relation with reACS was assessed by joint modelling. Results are reported as adjusted (for clinical factors) hazard ratios (aHRs).

Results

Median age was 64 (25th–75th percentiles; 56–72) years and 78% were men. After multiple testing correction ($p < 0.001$), high concentrations of extremely large very low density lipoprotein (VLDL) particles (aHR 1.60/SD increase; 95%CI 1.25–2.08), very large VLDL particles (aHR 1.60/SD increase; 95%CI 1.25–2.08) and large VLDL particles (aHR 1.56/SD increase; 95%CI 1.22–2.05) were significantly associated with reACS. Moreover, these longitudinal particle concentrations showed a steady increase over time prior to reACS. Among the other metabolites, no significant associations were observed.

Conclusion

Post-ACS patients with persistent high concentrations of extremely large, very large and large VLDL particles have increased risk of reACS within 1 year.

Introduction

In recent years, the rise of genomics has helped to unravel the human genome and to identify genes that are involved with the development of CVD[1]. However, CVD is a polygenic and multifactorial disease, which is both influenced by a patient's genetic predisposition, as well as affected by biological and chemical variation downstream of the genetic code. Whereas genomic research concentrates on the 'static' genotype of a patient, metabolomic research focuses on metabolites, which are the substrates or end-products of all enzymatic processes[2]. Metabolomic research creates a blueprint of a patient's metabolism at a specific time point and, accordingly, captures both the upstream influence of a patient's genotype as well as downstream variation influencing the metabolism[2]. Eventually, combining knowledge gained through metabolomic research with knowledge on genetics and clinical risk factors, may give rise to novel insights on the pathophysiology of CVD.

The number of longitudinal studies that have assessed the association between a patient's metabolite profile and development of CVD is increasing[3]. However, these studies relate single baseline measurements to the incidence of CVD events during long-term follow-up[3]. Since the metabolite profile of CVD patients is not a static given, but will likely be influenced by changes in disease severity over time, repeated metabolite profile measurements might carry incremental prognostic information over a single measurement.

We designed the 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) to study temporal biomarker changes in post-acute coronary syndrome (ACS) patients. The current report describes an analysis of the temporal patterns of 151 metabolites in these patients and the association of the repeatedly measured metabolites with reACS.

Methods

Study population

BIOMArCS is a multicenter observational study, conducted during 2008-2015 in the Netherlands. Details concerning the study design have been described elsewhere[4]. In brief, BIOMArCS enrolled patients who were admitted for ACS, either with or

without ST-elevation, and who had at least one additional CVD risk factor. After inclusion, venepuncture was performed at admission, discharge, and subsequently every two weeks during the first half-year and every four weeks during the second half-year. If logistic circumstances hindered inclusion during hospitalisation, patients could be included on the first outpatient visit within 6 weeks after discharge - the absence of early samples was then accepted. Samples were collected non-fasting.

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

Study design

BIOMArCS enrolled 844 patients, and 45 reached the study endpoint of reACS, defined as a cardiac death, non-fatal myocardial infarction (MI) or unstable angina (UA) requiring urgent coronary revascularization (endpoint cases). For reasons of cost-efficiency, we applied a case-cohort design with respect to the present metabolite analysis. A random sample of 150 patients was selected from the full cohort (which rendered 8 endpoint cases), and was complemented with the remaining 37 endpoint cases outside this random sample. Consequently, the case-cohort sample included all 45 study endpoint cases and 142 endpoint-free patients.

We realized that the metabolites could have been influenced by the index ACS event. We were mainly interested in metabolite patterns after clinical stabilization. Therefore, we restricted our analyses to the 28 study endpoint cases and 130 event-free patients with available blood samples after 30 days following the index event.

Metabolite analysis

Serum samples were collected and preserved on-site at -80 degrees Celsius. Subsequently, samples were transported to the Erasmus MC for long-term storage under the same conditions. For the current analysis, serum samples were analyzed applying high-throughput automated proton NMR spectroscopy by Nightingale Health[5]. In each blood sample, all metabolites were quantified simultaneously, and, subsequently, expressed in absolute concentrations using Nightingale Health's

proprietary software[5]. Details on the applied NMR method are described in the *supplemental material*. We assessed 151 metabolites, including 14 lipoprotein subclasses and their particle concentrations and lipids compositions, 9 cholesterol metabolites, 2 apolipoproteins, 8 glycerides and phospholipids, 9 fatty acids, 4 glycolysis related metabolites and 9 amino acids.

Statistical data analysis

Continuous variables are presented as median (25th-75th percentile). Categorical variables are presented as number (percentage). Differences in continuous data between study endpoint cases and event-free patients were evaluated by Mann-Whitney U tests, whereas categorical variables were evaluated by Pearson Chi-square tests.

The linear mixed effects (LME) model was used to describe the evolution of metabolites over time, with adjustment for age, gender, GRACE risk score, diabetes mellitus, history of peripheral arterial disease, statin use and vitamin K antagonist use. Cox proportional hazard regression was used to relate serially measured metabolite level, based on the LME model, with the incidence of the study endpoint, while adjusting for GRACE risk. The parameters of the LME and Cox models were estimated in a joint model to avoid bias[6]. To enable a direct comparison of the relation between different metabolites and the study endpoint, we present adjusted hazard ratios (aHR) as per one standard deviation (SD) difference.

R statistical software (version 3.4.3) was used for the statistical analyses, in particular the package JMbayes[6]. All statistical tests were two-tailed, and p-values <0.001 were considered statistically significant, to correct for multiple testing. This significance level was determined by matrix spectral decomposition[7].

Results

Median (25^{th}-75^{th} percentile) age was 63.8 (56.1-71.6) years and 77.8% were men. Study endpoint cases were older than event-free patients, had a higher prevalence of diabetes, history of peripheral arterial disease and vitamin K antagonist usage (Table 8.1), and had similar characteristics otherwise. For the current analysis, a

median (25^{th}-75^{th} percentile) of 7 (3-10) and 8 (5-9) repeated measurements were available in study endpoint cases and event-free patients, respectively.

Table 8.1 Baseline clinical characteristics.

	Overall	Event-free patients	Cases	p-value
No. patients	**158**	**130**	**28**	
Presentation and initial treatment				
Men	123 (77.8)	102 (78.5)	21 (75.0)	0.88
Age - yr	63.8 (56.1-71.6)	62.3 (55.1-71.0)	68.0 (59.0-76.3)	0.030
Admission diagnosis				0.59
STEMI	69 (43.7)	59 (45.4)	10 (35.7)	
NSTEMI	66 (41.8)	52 (40.0)	14 (50.0)	
UAP	23 (14.6)	19 (14.6)	4 (14.3)	
CAG performed	149 (94.3)	121 (93.1)	28 100.0)	0.33
PCI performed	124 (84.4)	100 (83.3)	24 (88.9)	0.67
Max CK - U/L	425.0 (179.0-1197.0)	452.5 (196.8-1200.8)	312.0 (135.0-750.5)	0.24
Cardiovascular risk factors				
Smoking				0.89
Current	65 (41.1)	54 (41.5)	11 (39.3)	
Former	48 (30.4)	40 (30.8)	8 (28.6)	
Never	45 (28.5)	36 (27.7)	9 (32.1)	
Diabetes mellitus	32 (20.3)	22 (16.9)	10 (35.7)	0.047
Hypertension	84 (53.2)	70 (53.8)	14 (50.0)	0.87
Hypercholesterolemia	76 (48.1)	66 (50.8)	10 (35.7)	0.22
Creatinine - µmol/L	82.5 (72.3-93.8)	82.0 (73.0-91.8)	86.5 (71.3-95.0)	0.46
Cardiovascular history				
Peripheral arterial disease	15 (9.5)	9 (6.9)	6 (21.4)	0.043
Myocardial infarction	51 (32.3)	42 (32.3)	9 (32.1)	1.00
PCI	47 (29.9)	37 (28.7)	10 (35.7)	0.61
CABG	16 (10.1)	11 (8.5)	5 (17.9)	0.25
Stroke	20 (12.7)	14 (10.8)	6 (21.4)	0.22
Valvular heart disease	5 (3.2)	2 (1.5)	3 (10.7)	0.055
Heart failure	7 (4.4)	4 (3.1)	3 (10.7)	0.20
Medication at first blood sample moment >7 days after the index ACS*				
Aspirin	150 (95.5)	122 (94.6)	28 (100.0)	0.45
P2Y12 inhibitor	145 (92.4)	118 (91.5)	27 (96.4)	0.62
Vitamin K antagonist	14 (8.9)	8 (6.2)	6 (21.4)	0.028
Statin	151 (96.2)	125 (96.9)	26 (92.9)	0.64
Beta-blocker	135 (86.0)	108 (83.7)	27 (96.4)	0.15
ACE inhibitor or ARB	131 (83.4)	105 (81.4)	26 (92.9)	0.23

*The first blood sample >7 days was taken at a median (25th-75th percentile) of 24 (16-34) days after the index ACS. Continuous variables are presented as median (25th-75th percentile). Categorical variables are presented as number (percentage). ACE: angiotensin converting enzyme, ARB: angiotensin II receptor blocker, CABG: coronary artery bypass grafting, CKmax: maximum creatinine kinase during the index admission, No: Numero, NSTEMI: non-ST-elevation myocardial infarction, PCI: percutaneous coronary intervention, STEMI: ST-elevation myocardial infarction, Troponinmax: maximum troponin value during the index admission, UAP: unstable angina pectoris, yr: year.

In addition, 95% of the 1101 samples were collected in patients on statins. Clinical characteristics did not significantly differ between statin-treated and statin-untreated patients (data not shown). LDL cholesterol was 0.46 (95%CI: 0.061-0.85) mmol/l per SD increase higher in the 55 samples collected in statin-untreated patients (p value = 0.024).

Metabolites

Higher concentrations of extremely large VLDL particles (XXL-VLDL-P), very large VLDL-P (XL-VLDL-P) and large VLDL-P (L-VLDL-P) were significantly associated with reACS (aHR 1.60/SD, 95% CI 1.25-2.08; aHR 1.60/SD, 95% CI 1.25-2.08; aHR 1.56/SD, 95% CI 1.22-2.05, respectively) during one year follow-up (Figure 8.1, Supplemental Table S8.1). Moreover, the concentrations of these particles steadily increased prior to the reACS (Figure 8.2).

In addition to the lipoprotein subclass particle concentrations, the lipid composition of each lipoprotein subclass was quantified with NMR (Supplemental Table S8.2). A lipoprotein particle is composed of phospholipids, cholesterol, cholesterol esters, free cholesterol and triglycerides. Figure 8.3 shows the aHR's of the lipid components of XXL-VLDL-P, XL-VLDL-P and L-VLDL-P. Overall, the individual lipid components of XXL-VLDL-P, XL-VLDL-P and L-VLDL-P were also associated with reACS. However, per lipid component we observed intra-variability (within the particle) and, more importantly, inter-variability (between the particles) in the degree of association with reACS. For instance, in XXL-VLDL the concentration of total cholesterol was associated with reACS with an aHR of 1.58/SD increase (95% CI: 1.18-1.94, p<0.001). In XL-VLDL, the concentration of total cholesterol had an aHR of 1.53/SD increase (95%CI: 1.19-1.97, p=0.006). In L-VLDL, the concentration of total cholesterol had an aHR of 1.34/SD increase (95% CI: 0.89-1.98, p=0.17).

Among the other assessed metabolite groups, no significant associations were identified between metabolite concentration and reACS.

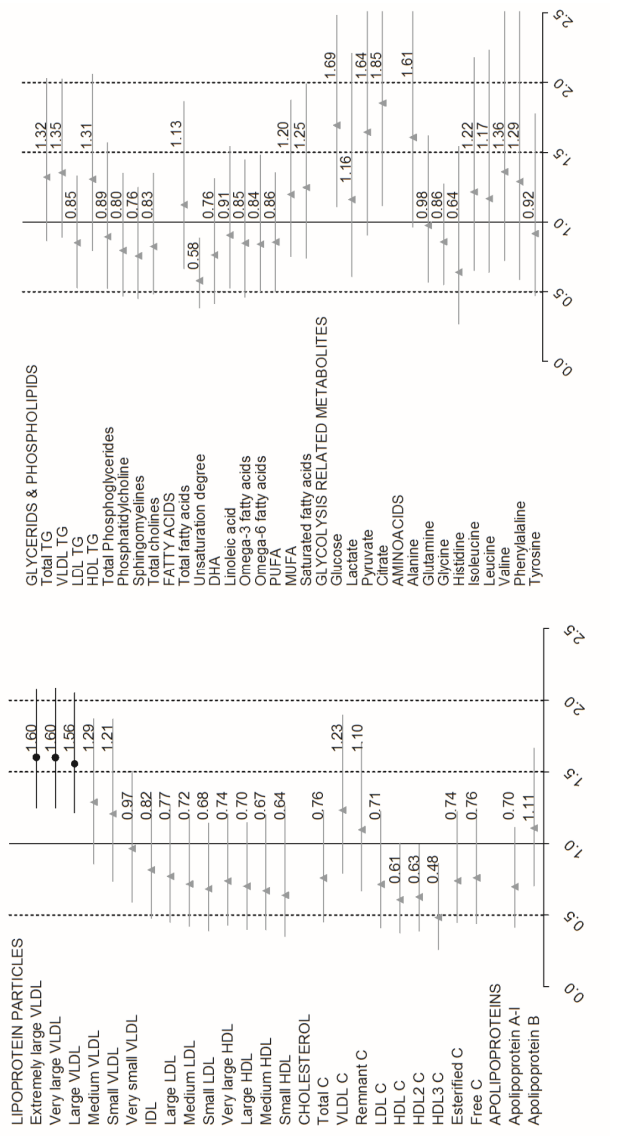
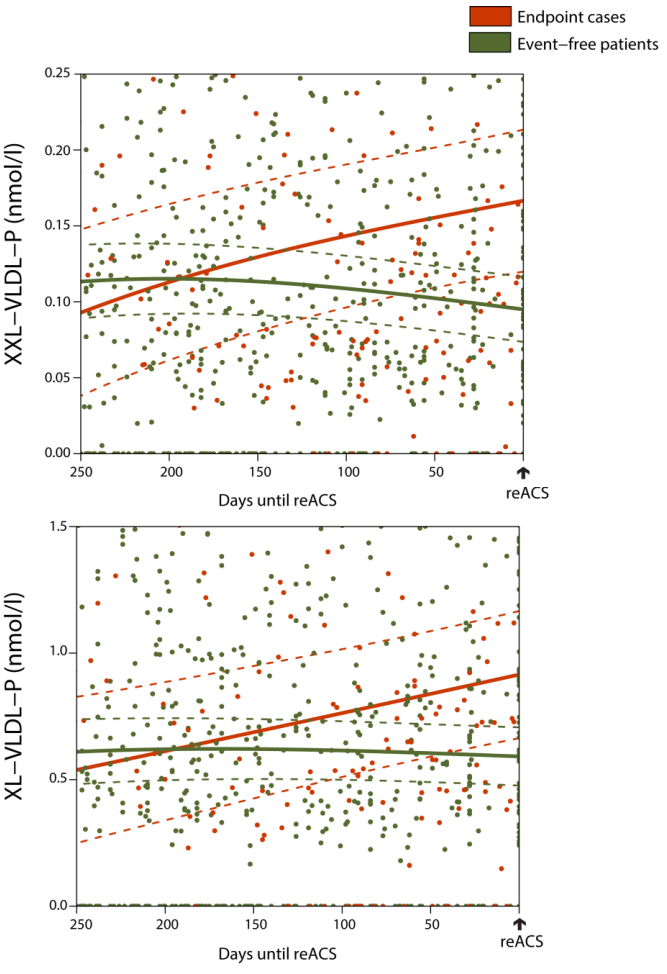


Figure 8.1 Associations of NMR-quantified metabolite profile and reACS. aHR's with 95%CI are presented on a SD-scale adjusted for age, gender, GRACE risk score, diabetes mellitus, history of peripheral arterial disease, statin use and vitamin K antagonist use. Black rounds are statistically significant with p<0.001, grey triangles are not. C: cholesterol, DHA: Docosahexaenoic acid, HDL: high density lipoprotein, IDL: intermediate density lipoprotein, LDL: low density lipoprotein, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty, TG: triglycerides, VLDL: very low density lipoprotein.


Endpoint cases
Event−free patients
XXL−VLDL−P (nmol/l)
0.25
0.20
0.15
0.10
0.05
0.00
250
200
150
100
50
reACS
Days until reACS
XL−VLDL−P (nmol/l)
1.5
1.0
0.5
0.0
250
200
150
100
50
reACS
Days until reACS

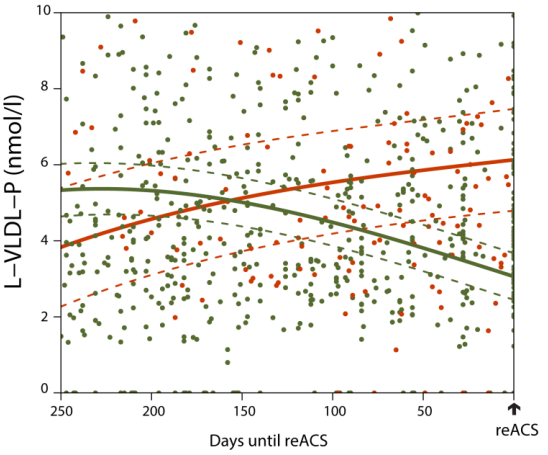


Figure 8.2 Longitudinal trajectory of XXL-VLDL-P, XL-VLDL-P and L-VLDL-P prior to reACS. nmol: nanomol, l: liter, L-VLDL-P: large VLDL particles, reACS: repeated acute coronary syndrome, XXL-VLDL-P: extremely large VLDL particles, XL-VLDL-P: very large VLDL particles.

Discussion

This study assessed the association between repeatedly measured metabolite profiles and the incidence of reACS during one year follow-up in post-ACS patients. Patients who experienced reACS had steadily increasing concentrations of XXL-VLDL-P, XL-VLDL-P and L-VLDL-P during one-year of follow-up until the moment of the endpoint event. No significant associations were detected between longitudinal serum concentrations of cholesterol metabolites, apolipoproteins, glycerides and phospholipids, fatty acids, glycolysis related metabolites or amino acids and reACS. Hence, serial blood sampling may benefit the prognostic accuracy of lipoprotein particle concentrations over a single baseline measurement. In a larger study cohort with more patients developing cardiac outcome, one should assess the frequency of sampling needed for accurate prognostication using lipoprotein particle concentrations.

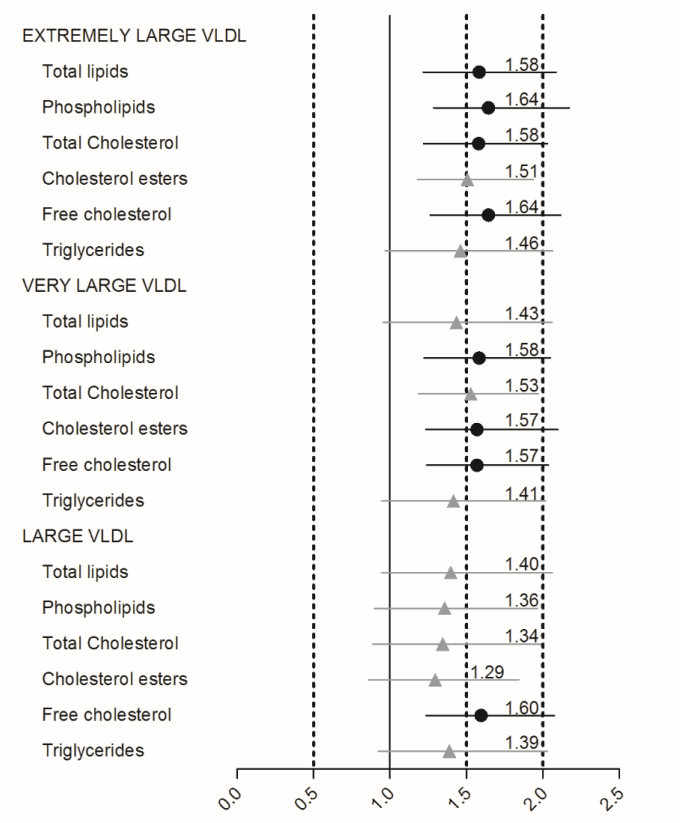


Figure 8.3 Associations of NMR-quantified components of extremely large, very large and large VLDL particles and reACS. aHR's with 95%CI are presented on a SD-scale adjusted for age, gender, GRACE risk score, diabetes mellitus, history of peripheral arterial disease, statin use and vitamin K antagonist use. Black rounds are statistically significant with p<0.001, grey triangles are not. VLDL: very low density lipoprotein

Our study predominantly consisted of statin-treated patients. Previously, Würtz et al. showed in a combined analysis of population-based cohorts, that statin-use lowered most of their NMR-quantified metabolite concentrations[8]. In particular, statins effectively lowered multiple lipoprotein concentrations in addition to LDL cholesterol. In our study, despite statin use, XXL-VLDL-P, XL-VLDL-P and L-VLDL-P concentrations were significantly higher in patients who experienced a reACS, whereas total VLDL cholesterol was not. Since recent years, studies are advocating

the added value of lipoprotein particle concentrations to lipoprotein cholesterol concentrations for clinical prognosis in patients with CVD[9]. Moreover, in 2011, the American National Lipid Association Expert panel has advised to study the use of lipoprotein particle concentrations to enhance treatment management, to address the residual risk of statin-treated patients with CVD for adverse outcome[10]. Subsequently, several studies have found that LDL particle concentration is a better predictor of adverse outcome than LDL cholesterol in CVD patients on lipid-lowering treatment[9]. One can argue that the latter finding might also be true for VLDL. It has been previously described that elevated VLDL cholesterol levels are an independent predictor of adverse outcome in the general population and in patients with CVD, and it has been suggested that VLDL cholesterol in combination with LDL cholesterol may be a better determinant of adverse outcome than LDL cholesterol alone[11-13]. In our current study, we found that the larger VLDL particle concentrations were associated with reACS, whereas total VLDL cholesterol was not. Hence, further research should establish if VLDL particle concentrations provide incremental prognostic information to LDL particle concentrations in statin-treated CVD patients to address their risk of developing adverse outcome.

Although not significant, plasma glucose appeared to correlate with reACS in our study. Previously, it has been demonstrated that hyperglycemia induces overproduction of larger VLDL particles[14]. Thus, potentially, the post-ACS patients who experienced reACS had a certain grade of hyperglycemia which may have induced the overproduction of larger VLDL particles and subsequent pathological atherogenesis.

Currently, results obtained by metabolite profiling are difficult to compare across various study populations, due to lack of a uniform way to quantify metabolites and otherwise heterogeneous study methods[3]. Although NMR is a cost-effective tool to obtain detailed knowledge on metabolites[15], the sensitivity of this technique is limited compared with other metabolite profiling techniques such as mass spectrometry. Still, also mass spectrometry has downsides, including automation of the technique and the fact that it cannot detect lipoproteins[15]. Therefore, in our view, NMR suits purposes of epidemiological studies including ours, whereas mass spectroscopy is more suited for detailed metabolite discovery. Eventually, the field of CVD metabolite research should focus on developing uniform study methods, as well as profiling techniques to obtain more reliable and comparable results. Under

such conditions, the knowledge that will be gained through metabolite profiling might enable a precision-medicine approach to CVD treatment.

Limitations

The current study utilized 1101 serial blood samples, to assess the time course of NMR-quantified metabolites and their longitudinal association with incident ACS. Nonetheless, as only 28 study endpoint cases were available, we cannot exclude the possibility that our study was underpowered. In addition, freezing and thawing of serum samples could have influenced the metabolite measurements. However, our samples were kept frozen at -80°C throughout complete storage and transportation of the samples up until quantification. Lastly, because of the exploratory character of our study, we could not provide a mechanical interpretation of our findings.

Conclusion

Post-ACS patients with persistent high concentrations of extremely large, very large and large VLDL particles have increased risk of reACS within 1 year.

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Part III

Prognostic value of repeated echocardiograpms and repeated blood biomarker measurements in chronic heart failure

Chapter 9

Repeated echocardiograms do not provide incremental prognostic value to single echocardiographic assessment in minimally symptomatic patients with chronic heart failure: Results of the Bio-SHiFT study

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Journal of the American Society of Echocardiography. 2019

Abstract

Background

We aimed to compare the prognostic value of a single 'baseline' echocardiogram with repeated echocardiography in stable chronic heart failure (CHF) patients. We hypothesized that repeated echocardiograms would contain incremental prognostic information.

Methods

In the prospective Bio-SHiFT study, we performed 332 echocardiograms in 106 patients during a median follow-up of 2.3 years. The endpoint comprised HF hospitalization, left-ventricular assist device implantation, heart transplantation, and cardiovascular death. We compared hazard ratios (HRs; adjusted for NT-proBNP) from Cox models for the first available measurement with HRs from joint models, which model individual trajectories based on the repeated measurements and link these to the time-to-event data.

Results

Mean age was 58.1 years, 78.3% were male, 12.6% had NYHA-class >II, all had reduced ejection fraction (rEF) and most common HF etiologies were cardiomyopathies (51%) and ischemia (40%). The endpoint occurred in 25 patients. Both the single measurements and the temporal trajectories were significantly associated with the endpoint (adjHR Cox model (95%CI) vs. adjHR joint model (95%CI)): Left ventricular (LV) ejection fraction: 1.47(0.93-2.31) vs. 1.77(1.13-2.93), diastolic LV diameter: 1.64(1.09–2.47) vs. 1.68(1.12-2.57), systolic LV diameter: 1.72(1.10-2.69) vs. 1.68(1.13-2.63), systolic left atrial diameter: 1.88(1.18-3.00) vs. 2.60(1.48-4.97), E/A-ratio 2.73(1.42-5.26) vs. 3.87(1.75-10.13), and E/e'-ratio 2.30(1.38-3.84) vs. 2.99(1.68-6.19). None of the trajectories from the investigated parameters showed worsening prior to events.

Conclusion

Although single baseline or repeatedly measured echocardiographic parameters were associated with the endpoint, all parameters remained on average stable during the 2.3 years follow-up in this, largely, minimally symptomatic CHF cohort. Thus, regular echocardiographic monitoring of systolic or diastolic LV function within this time-frame does not carry incremental prognostic information over a single baseline measurement.

Introduction

Echocardiography plays a central role in chronic heart failure (CHF) and is used on a daily basis for diagnosis and follow-up[1]. Echocardiography is relatively inexpensive, has a high feasibility and is capable of producing robust and simple measurements, such as left ventricular ejection fraction (LVEF), the dimensions of the left ventricle and atria, and the ratio of peak early (E) and late (A) filling velocities. For many of these measurements, studies have shown that they carry prognostic value in patients with heart failure[2-6]. However, in these studies, single, baseline echocardiographic measurements were related to the clinical endpoints of interest. Single measurements merely provide a snapshot of a patient's condition and fail to identify high-risk periods in individual patients. We hypothesize that repeating echocardiography may provide incremental insights into individual temporal patterns of systolic and diastolic function in CHF patients, and may herewith help identify periods in which an individual is at high risk of an event. If our hypothesis is confirmed and such periods exist, we could use dynamic predictions for updating the current risk of an event after each new echocardiogram, leading to an individual and up-to-date risk assessment which could be helpful for treatment adjustment[7].

To investigate our hypothesis, we compared the prognostic value of a single 'baseline' echocardiogram with the prognostic value of repeated echocardiograms in clinically stable patients with CHF. In addition, we investigated if the repeatedly measured echocardiographic parameters have incremental prognostic value over repeatedly measured N-terminal pro-Brain Natriuretic peptide (NT-proBNP).

Materials and methods

Details on the design of the Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT) study have been published previously[8]. In short, Bio-SHiFT is a prospective, observational cohort of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Northwest clinics, Alkmaar, The Netherlands. Patients were recruited during their regular outpatient visits while in clinically stable condition (i.e. they had not been hospitalized for HF in the three months prior to inclusion). The main inclusion criterion was a diagnosis of HF

according to the guidelines of the European Society of Cardiology three or more months before inclusion[9]. We excluded patients younger than 18 years.

After inclusion, the patients were followed for a maximum duration of 30 months, during which study follow-up visits were scheduled every three months (a window of +/- one month was allowed). At each follow-up visit, a short medical evaluation was performed, blood samples were drawn, and occurrence of adverse cardiovascular events since the previous visit were recorded. During the study, the routine outpatient follow-up by the treating physician continued for all patients, independently of the study visits. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). All patients signed informed consent for their participation in the study.

Three hundred ninety-eight patients were included in Bio-SHiFT. In this repeated echo substudy, we aimed to include 100 patients. The substudy was only performed at Erasmus MC, and consisted of repeated echocardiograms performed every six months during follow-up, additional to the tri-monthly blood sampling in Bio-SHiFT.

Echocardiography measurements and evaluation

Two-dimensional grayscale harmonic images were obtained in the left lateral decubitus position using a commercially available ultrasound system (iE33, Philips, Best, The Netherlands), equipped with a broadband (1-5 MHz) S5-1 transducer (frequency transmitted 1.7MHz, received 3.4 MHz) and stored in the echo core lab of Erasmus MC. Using specialized software (TOMTEC imaging, Unterschleissheim, Germany), the following parameters were measured: left ventricular ejection fraction (LVEF, using triplane), end-diastolic and end-systolic LV diameter, and end-systolic left atrial diameter[10]. The vena cava inferior diameter (including the results of the 'sniff test'), the tricuspid regurgitation (TR) velocity and the function of the aortic, mitral, and tricuspid valve, were also assessed but currently not evaluated in the longitudinal models.

The diastolic parameters were evaluated using Philips Excellera version R4.1 (Philips medical systems, the Netherlands). In order to assess diastolic function, the E/A ratio and the ratio of the E and early diastolic mitral annular velocity (e') were

calculated. For the e', we used the average of the lateral and medial e' when available; however, if only one of the two was available, this value was used.

All echocardiographic measurements were performed blinded to biomarker and clinical event data.

NT-proBNP measurement

Blood samples were processed and stored at a temperature of -80°C within 2 hours after blood collection. To determine NT-proBNP levels, a batch analysis was performed using an electrochemiluminescence immunoassay (Elecsys 2010; Roche Diagnostics, Indianapolis, IN). Accordingly, results of the biomarker assays were not available to treating physicians at the time of the outpatient visits and did not interfere with usual care.

Clinical study endpoints

The endpoint comprised the composite of hospitalization for the management of acute or worsened HF, left ventricular assist device implantation, cardiac transplantation, and cardiac death, whichever occurred first in time. All events were adjudicated by a clinical event committee blinded to the echocardiographic assessments and biomarker measurements, after reviewing corresponding hospital records and discharge letters.

Statistical analyses

Distributions of continuous variables were tested for normality using the Shapiro-Wilk test. Normally distributed continuous variables are presented as mean ± standard deviation (SD), and non-normally distributed variables as median and interquartile range (IQR). Categorical variables are presented as numbers and percentages. Differences in baseline characteristics between patients that experienced the endpoint and those who did not were tested using t-test and Mann-Whitney test respectively for continuous variables, and chi square tests and Fisher's exact tests for categorical variables.

We first evaluated the association between the echo parameters from the first available echocardiogram with the time until the occurrence of the composite

endpoint or censoring using a Cox proportional hazard model corrected for baseline NT-proBNP. Hereafter, we assessed the incremental value of repeated echocardiographic measurements. For this purpose, we used the framework of joint models for longitudinal and survival data. In these joint models, a linear mixed-effects (longitudinal) model provided estimates of the individual temporal trajectories for each echo parameter, while accounting for the correlation in the repeated measurements. These estimated trajectories were then combined with a Cox proportional hazards model, to study their association with the risk of the study endpoint. The individual trajectories, resulting from the linear mixed models, were adjusted for all variables (age, sex, renal failure and heart rate) for whom the p-value for the difference between those reaching the endpoint and those remaining endpoint-free was <0.1. The associations between the temporal evolutions and the endpoint, resulting from the Cox model, were firstly adjusted for baseline NT-proBNP levels. In a second series of multivariable joint models, we combined the repeatedly performed echocardiograms with repeatedly measured NT-proBNP, in order to investigate the incremental value of repeated echoes when repeated biomarker measurements are also available.

In order to compare effect sizes of different variables, all investigated echo parameters and the NT-proBNP measurements were firstly transformed to achieve a normal distribution after which the corresponding Z-score was calculated. This standardization was performed on the entire dataset (containing all repeated measurements)., Hereafter, to obtain the HRs entailed by the first echoes only, first echoes were selected and entered into Cox models; whilst to obtain the HRs entailed by the repeatedly measured echoes, joint models were performed on all available echoes. Thus, the results of the regression analyses of the Cox and joint models can be directly compared and are presented as hazard ratios (HR) which represent risk per SD increase/decrease of the standardized variable, along with the corresponding 95% confidence intervals (CI). As interpretation of the unit of these HRs may not be straightforward, we back-transformed the mean and +/- SD of the transformed variables and depicted these values in the tables.

As described above, our aim was to investigate whether repeatedly assessed echocardiographic parameters carry incremental predictive value to repeatedly measured NT-proBNP. We chose to present our results solely as hazard ratios adjusted for NT-proBNP and not combine them with C-statistics. Pepe et al. have

demonstrated that testing for improvement in prediction performance is actually redundant if a variable has already been shown to be an independent risk factor, and that standard testing procedures for C-indices are very conservative and thus insensitive to improvements in prediction performance[11].

Missing values in echo parameters were, except for the A wave, always due to poor image quality and were as such missing completely at random. Accordingly we chose to perform a complete case analysis. Missing values for the A wave were mostly due to atrial fibrillation during the echo or due to mitral valve replacement or clipping. In this specific patient group imputation of missing values is inappropriate, as the A wave can never be measured. Thus, we again chose for a complete case analysis here. The results of this analysis should not be extrapolated to patients excluded from the analysis.

All analyses were performed with R Statistical Software using packages 'nlme'[12] and 'JMbayes'[13]. All tests were two-tailed and p-values <0.05 were considered statistically significant.

Results

Baseline characteristics and clinical endpoints

From 2011 to 2017, 106 patients were included in the echocardiography substudy. All patients had reduced ejection fraction (rEF). Specifically, all patients in whom EF was measured by the automated software had EF <50%. In 2 patients, EF could not be measured in the first available echo by the automated software due to reduced image quality; based on the available images EF in these patients was classified as poor. A summary of the baseline characteristics is displayed in Table 9.1. The majority of the included patients were men (78.3%), the mean age was 58 ±11 (SD) years and 40% had HF due to ischemic disease.

Table 9.1 Baseline patient characteristics in relation to the occurrence of the composite endpoint.

		Composite endpoint reached		
Variable	**Total**	**No**	**Yes**	**p value**
N	106	81	25	
Demograhics				
Age, years (mean (SD))	58.1 (10.7)	57.8 (10.4)	59.3 (11.7)	0.56
Men, n (%)	83 (78.3)	63 (77.8)	20 (80.0)	1.00
Clinical characteristics, (mean (SD))				
BMI, kg/m^2 (median (IQR))	26.8 (24.2-30.6)	26.8 (24.1-31.3)	26.5 (24.2-29.2)	0.46
Heart rate, bpm (mean (SD))	65 (9)	64 (9)	66 (9)	0.05
Systolic blood pressure, mmHg (mean (SD))	110 (19)	111 (19)	105 (19)	0.14
Diastolic blood pressure, mmHg (median (IQR))	70 (60-79)	70 (61-79)	65 (60-70)	0.19
Features of heart failure				
NYHA class, n (%)				0.30
NYHA class I	32 (31.1)	27 (34.2)	5 (20.8)	
NYHA class II	58 (56.3)	43 (54.4)	15 (62.5)	
NYHA class III	13 (12.6)	9 (11.4)	4 (16.7)	
LVEF, % (mean (SD))	27 (9)	29 (9)	23 (6)	0.02
NT-proBNP, pmol/L (median (IQR))	124.7 (37.5. 219.3)	86.0 (27.3. 192.5)	235.0 (139.6. 422.0)	<0.01
Etiology of heart failure, n (%)				
Ischemic	40 (39.6)	30 (38.5)	10 (43.5)	0.85
Hypertension	2 (2.1)	2 (2.7)	0 (0.0)	1.00
Secondary to valvular disease	3 (3.1)	1 (1.3)	2 (9.5)	0.23
Cardiomyopathy	51 (52.0)	39 (52.0)	12 (52.2)	1.00
Other	13 (13.5)	11 (14.7)	2 (9.5)	0.80
Unkown	6 (6.8)	5 (7.4)	1 (5.0)	1.00
Medical history, n (%)				
Time since first HF episode, years (median(IQR))	5.7 (2.2-10.2)	5.1 (2.2-9.4)	7.6 (4.3-11.6)	0.20
Prior MI	39 (36.8)	29 (35.8)	10 (40.0)	0.89
Prior PCI	36 (34.3)	27 (33.8)	9 (36.0)	1.00
Prior CABG	8 (7.5)	6 (7.4)	2 (8.0)	1.00
Atrial fibrillation	27 (25.7)	17 (21.2)	10 (40.0)	0.11
Diabetes	26 (24.5)	19 (23.5)	7 (28.0)	0.85
Renal failure	42 (40.0)	26 (32.1)	16 (66.7)	<0.01
COPD	12 (11.4)	10 (12.3)	2 (8.3)	0.86
Medication use, n (%)				
Beta-blocker	102 (97.1)	79 (97.5)	23 (95.8)	1.00
ACE-Inhibitor	77 (74.0)	59 (73.8)	18 (75.0)	1.00
ARB	26 (24.8)	22 (27.2)	4 (16.7)	0.44
Loop diuretics	96 (92.3)	72 (90.0)	24 (100.0)	0.24
Aldosterone antagonist	68 (65.4)	51 (63.7)	17 (70.8)	0.69

n: number; SD: standard deviation; IQR: interquartile range; BMI: body mass index; bpm: beats per minute; mmHg: Millimeter of mercury; NYHA: New York Heart Association; LVEF: left ventricular ejection fraction; NT-proBNP: N-terminal pro brain natriuretic peptide; pmol/L: picomole per liter; HF: heart failure; MI: myocardial infarction; PCI: percutaneous coronary intervention; CABG: coronary artery bypass grafting; COPD: chronic obstructive pulmonary disease; ACE: angiotensin converting enzyme; ARB: Angiotensin receptor blocker

In total 25 (23.6%) patients reached the combined endpoint; 18 patients were re-hospitalized for acute or worsened heart failure; 2 patients received a left-ventricular assist device implantation, 2 patients received a heart transplantation, and 3 patients died from cardiovascular causes. Patients who later experienced an endpoint had a higher baseline heart rate, NT-proBNP, and were more likely to have renal failure than their counterparts who remained endpoint-free.

Echocardiography

In the 106 HFrEF patients, a total of 332 echocardiograms were performed with a median (IQR) of 3 (2-4) echocardiograms per patient during a median (IQR) follow-up time of 2.3 (1.7-2.7) years. Missings mostly occurred due to logistic circumstances (e.g. the unavailability of an ultrasound technician during the study visit). The median time between the last echo and the moment that the event or censoring occurred was 69 (37-165) days in patients in whom the event occurred and 180 (92-280) days in event-free patients.

Echocardiographic parameters were successfully measured in more than 90% of available echocardiograms except for E/A ratio. Due to atrial fibrillation and severe mitral valve disease, we were only able to determine E/A-ratio from 81.3% of the echoes (270 echoes, 94 patients, 20 events). At least one measurement of each of the other repeatedly assessed parameters was available in all 106 patients, except for end-systolic left atrial diameter and E/e'-ratio, which were missing completely in one patient.

First available echocardiogram

Table 9.2 displays the characteristics of the first available echocardiogram for each included patient. Because of logistic reasons, seventy-two percent of these echoes were performed during the baseline visit (follow-up time zero), 14.2% during the first follow-up visit (target follow-up time 3 months), 8.5% during the second follow-up visit (target 6 months), and the remaining 5.7% hereafter.

Patients who experienced the endpoint had a lower LV ejection fraction and larger LV and atrial dimensions than patients who remained endpoint-free. In addition, those who experienced an endpoint had higher E/A-ratios, E/e'-ratios, and TR-velocities and were more likely to have (severe) mitral valve regurgitation and

tricuspid valve regurgitation. All echocardiographic parameters except for LVEF, were significantly associated with the study endpoint, independently of the baseline NT-proBNP (Table 9.3). Although the estimate did not reach statistical significance for LVEF, it was substantial (HR (95%CI): 1.47 (0.93-2.31)). The E/A ratio and the E/e' ratio (available in 97 of 106 patients), were the strongest predictors with hazard ratios per SD increase of 2.73 (95%CI 1.42-5.26) and 2.30 (95%CI 1.38-3.84) respectively.

Table 9.2 Echocardiographic characteristics from first available echo in relation to the occurrence of the composite endpoint.

	N of echos in which variable was measured (%)	Total 106	Composite endpoint reached: No 81	Composite endpoint reached: Yes 25	p value
Systolic parameters					
LVEF (mean (sd))	104 (98.1)	28 (9)	30 (9)	22 (7)	<0.001
DiasLVD (median (IQR))	101 (95.3)	64 (59, 73)	63 (58, 69)	73 (64, 79)	0.001
SysLVD (median (IQR))	99 (93.4)	57 (49, 64)	53 (46, 62)	63 (56, 72)	0.001
SysLAD (mean (sd))	97 (91.5)	43.28 (8.64)	41.38 (7.80)	49.39 (8.52)	<0.001
Diastolic parameters (median (IQR))					
E/A ratio	83 (78.3)	1.10 (0.80, 1.88)	0.99 (0.73, 1.24)	2.38 (1.29, 3.38)	<0.001
E/E' ratio	97 (91.5)	13.2 (9.7, 18.3)	11.0 (9.0, 14.7)	22.9 (17.9, 27.8)	<0.001
TR velocity	70 (66.0)	2.50 (2.24, 2.86)	2.42 (2.14, 2.65)	2.89 (2.44, 3.32)	0.005
Vena Cava					
VCI (median (IQR))	84 (79.2)	16 (12, 19.25)	15 (12, 19)	20 (11, 22)	0.062
Valvular dysfunction (%)					
Mitral valve regurgitation	101 (95.3)				0.002
none		31 (30.7)	29 (37.7)	2 (8.3)	
mild		41 (40.6)	29 (37.7)	12 (50.0)	
moderate		22 (21.8)	17 (22.1)	5 (20.8)	
severe		7 (6.9)	2 (2.6)	5 (20.8)	
Aorta valve regurgitation	101 (95.3)				0.121
none		90 (89.1)	71 (92.2)	19 (79.2)	
mild		8 (7.9)	5 (6.5)	3 (12.5)	
moderate		3 (3.0)	1 (1.3)	2 (8.3)	
Tricuspid valve regurgitation	101 (95.3)				0.004
none		55 (54.5)	46 (59.7)	9 (37.5)	
mild		37 (36.6)	28 (36.4)	9 (37.5)	
moderate		3 (3.0)	2 (2.6)	1 (4.2)	
severe		6 (5.9)	1 (1.3)	5 (20.8)	

P-values were based on T-test or Mann-Whitney test for continuous variables depending on their distribution. To test for differences in the categorical variables, chi-square tests were performed. N: number; LVEF: left ventricular ejection fraction; sd: standard deviation; IQR: interquartile range; DiasLVD: diastolic left ventricular diameter; SysLVD: systolic left ventricular diameter; SysLAD: systolic left atrial diameter; E: peak early filling velocity; A: peak late filling velocity; e': early diastolic mitral annular velocity; TR: tricuspid regurgitation; VCI: inferior vena cava

Table 9.3 Results from first available echo.

Variable	N of patients (N of events)	mean ± SD	HR (95%CI)	p value
Left ventricular Ejection fraction*	104 pt (24)	29 (20, 38)	1.47 (0.93 - 2.31)	0.101
Diastolic left ventricular diameter†	101 pt (24)	65 (55, 77)	1.64 (1.09 - 2.47)	0.017
Systolic left ventricular diameter†	99 pt (24)	56 (45, 69)	1.72 (1.10 - 2.69)	0.017
Systolic left atrial diameter†	97 pt (23)	42 (35, 51)	1.88 (1.18 - 3.00)	0.008
E/A-ratio‡	83 pt (20)	1.00 (0.65, 2.00)	2.73 (1.42 - 5.26)	0.003
E/e'-ratio†	97 pt (24)	12.9 (7.4, 22.5)	2.30 (1.38 - 3.84)	0.001

HRs represent change in risk of the endpoint for a 1 standard deviation change in the echo parameter at any point in time during follow-up. All models are corrected for baseline NTproBNP. * HR per one SD decrease. † HR per one SD increase on the log2 scale. ‡ HR per one SD increase after ^.66 transformation.

Repeated echocardiograms

The individual trajectories of all of the investigated echo parameters were significantly associated with the clinical endpoint independently of baseline NT-proBNP. The corresponding HRs were comparable, or slightly larger, to those found for the first available measurements (Table 9.4). In line with the results from the 'single measurement' analysis, E/A-ratio and E/e' ratio showed the greatest HR per SD increase.

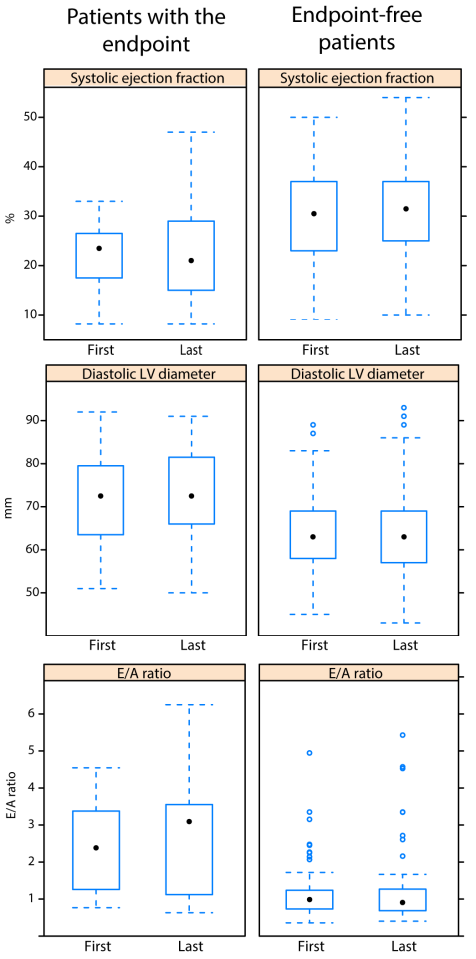
Table 9.4 Results for repeatedly measured parameters.

Variable	N of available echos (N of patients, N of events)	mean ± SD	HR (95%CI)	p value
Left ventricular Ejection fraction*	327 (106 pt, 25 events)	29 (20, 38)	1.77 (1.13-2.93)	0.017
Diastolic left ventricular diameter†	325 (106 pt, 25 events)	65 (55, 77)	1.68 (1.12-2.57)	0.012
Systolic left ventricular diameter†	320 (106 pt, 25 events)	56 (45, 69)	1.68 (1.13-2.63)	0.011
Systolic left atrial diameter†	316 (105 pt, 24 events)	42 (35, 51)	2.60 (1.48-4.97)	0.001
E/A-ratio‡	270 (94 pt, 20 events)	1.0 (0.65, 2.0)	3.87 (1.75-10.13)	0.001
E/e'-ratio†	311 (105 pt, 25 events)	12.9 (7.4, 22.5)	2.99 (1.68-6.19)	<0.001

HRs represent change in risk of the endpoint for a 1 standard deviation change in the echo parameter at any point in time during follow-up. * HR per one SD decrease. † HR per one SD increase on the log2 scale. ‡ HR per one SD increase after ^.66 transformation. The survival part of the models, captured by the Cox-model, were corrected for NTproBNP at baseline. In addition the trajectories of the biomarkers, captured by the linear mixed model, were corrected for age, sex, renal failure, and heart rate. N: Number; HR: HR ratio; SD: standard deviation; pt: patient; E:peak early filling velocities; A: peak late filling velocities.

Although the repeatedly measured echo parameters were associated with occurrence of the endpoint, we could not identify any increase or decrease of their

average temporal patterns as the endpoint approached (Figure 9.1 & Figure 9.2). The average trajectories of the echo parameters remained stable as the composite endpoint or the moment of censoring approached.



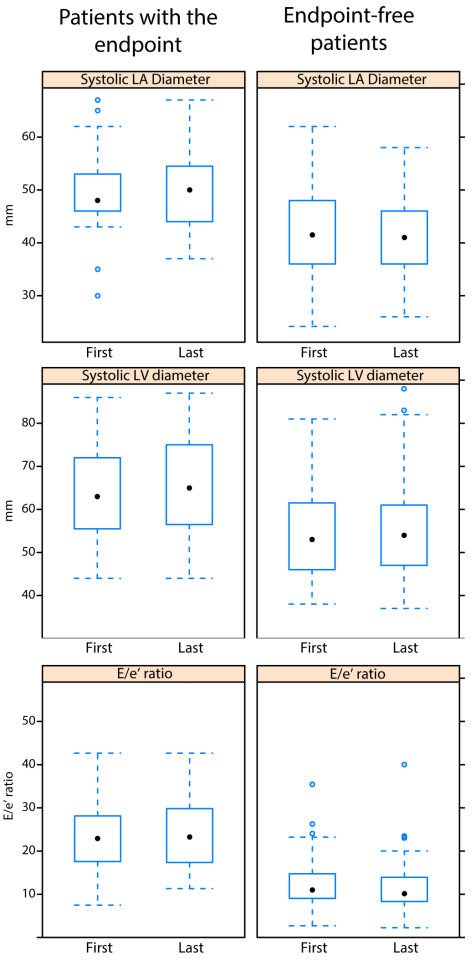


Figure 9.1 Average levels of echocardiographic parameters at the first and last measurement. The results of each of the investigated echo parameters are depicted in two paired boxplots. In these pairs, the left boxplot shows the average echocardiographic parameter levels in patient with the endpoint; while the right boxplot shows the average biomarker levels in endpoint-free patients. Within each boxplot, the left box represents the average of the first 'baseline' measurement. The right box depicts the average of the last available measurement for each patient. LV: left ventricular; LA: left atrial; E: peak early filling velocity; A: peak late filling velocity; e': early diastolic mitral annular velocity; mm: millimeter.

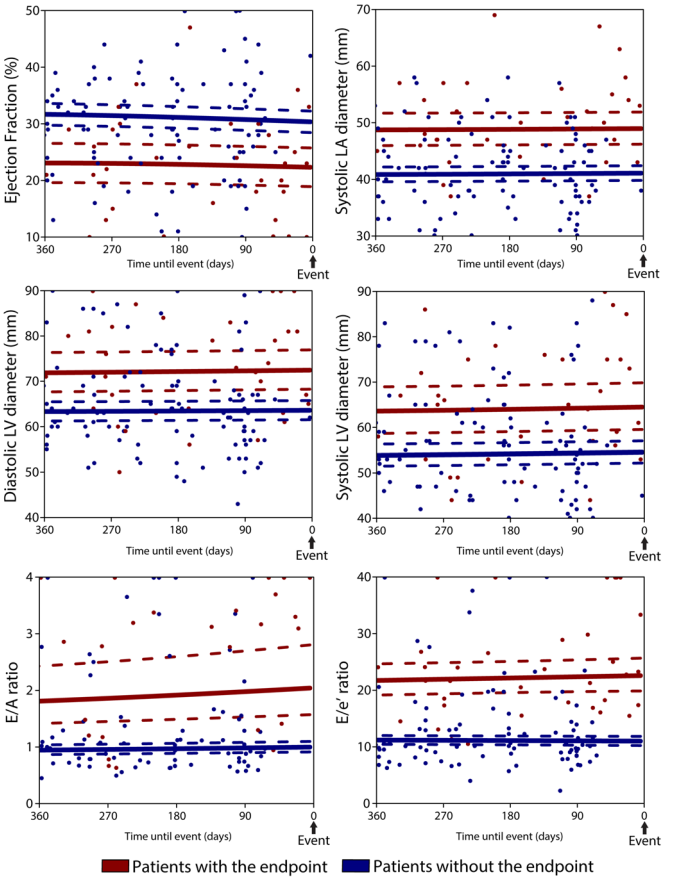


Figure 9.2 Average levels of echo parameters until time of event. The X-axis represents the time remaining to the primary endpoint (for patients who experienced incident adverse events) or time remaining to censoring (for patients who remained event-free). Of note is that 'time zero' is defined as the occurrence of the endpoint and is depicted on the right side of the x-axis, so that the average echocardiographic parameter can be visualized as the endpoint approaches. The continuous lines represent the average temporal pattern for patients with the endpoint (red) and endpoint-free patients (blue) as extracted from the joint model. The corresponding dotted lines depict the 95% confidence intervals. Finally, each dot represents a single measurement. LV: left ventricular; LA: left atrial; E: peak early filling velocity; A: peak late filling velocity; e': early diastolic mitral annular velocity; mm: millimeter.

Repeated echocardiograms and NT-proBNP measurements combined

During follow-up of the 106 patients a total of 819 (median 8 (IQR 7-10) per patient) blood samples were taken for NT-proBNP measurement. The results of the multivariable joint models in which both the repeatedly measured NT-proBNP and the repeatedly measured echo parameters were entered are shown in Table 9.5. The HRs of all of the echo parameters somewhat decreased compared to the joint model adjusted solely for baseline NT-proBNP. Although repeatedly measured NT-proBNP was a much stronger predictor of the composite endpoint, all repeatedly assessed echo parameters except for LVEF were independently associated with the endpoint, and herewith clearly provided incremental prognostic value. LVEF was not an independent predictor but the corresponding estimate was still substantial (HR (95%CI): 1.52 (0.95-2.45)). Notably, of all investigated echo parameters, the E/A-ratio and the E/e'-ratio again were again the strongest predictors of the composite endpoint.

Table 9.5 Results for repeatedly measured parameters, adjusted for repeatedly measured NT-proBNP.

Variable	N of available echos (N of patients N of events)	mean ± SD	HR (95%CI)	p value
Left ventricular Ejection fraction*	327 (106 pt, 25 events)	29 (20, 38)	1.52 (0.95 - 2.45)	0.076
NT-proBNP†		84.4 (21.3, 334.2)	6.47 (3.24 - 13.60)	<0.001
Diastolic left ventricular diameter†	325 (106 pt, 25 events)	65 (55, 77)	1.56 (1.04 - 2.38)	0.026
NT-proBNP†		84.4 (21.3, 334.2)	7.53 (3.67 -15.54)	<0.001
Systolic left ventricular diameter†	320 (106 pt, 25 events)	56 (45, 69)	1.53 (1.05 - 2.41)	0.032
NT-proBNP†		84.4 (21.3, 334.2)	7.39 (3.60 - 15.76)	<0.001
Systolic left atrial diameter†	316 (105 pt, 24 events)	42 (35, 51)	2.02 (1.15 - 3.76)	0.008
NT-proBNP†		84.4 (21.3, 334.2)	6.65 (3.35 - 14.15)	<0.001
E/A-ratio‡	270 (94 pt, 20 events)	1.0 (0.65, 2.0)	2.70 (1.16 - 8.32)	0.026
NT-proBNP†		84.4 (21.3, 334.2)	7.59 (3.16 - 19.82)	<0.001
E/e'-ratio†	311 (105 pt, 25 events)	12.9 (7.4, 22.5)	2.51 (1.22 - 5.68)	0.012
NT-proBNP†		84.4 (21.3, 334.2)	5.00 (2.22 - 11.53)	0.002

HRs represent change in risk of the endpoint for a 1 standard deviation change in the echo parameter at any point in time during follow-up. * HR per one SD decrease. † HR per one SD increase on the log2 scale. ‡ HR per one SD increase after ^.66 transformation. The trajectories of both the echo biomarker and NT-proBNP, captured by the linear mixed model, were corrected for age, sex, renal failure and heart rate.. N: Number; HR: HR ratio; SD: standard deviation; pt: patient; E:peak early filling velocities; A: peak late filling velocities; NT-proBNP: N-terminal pro-Brain Natriuretic peptide.

Discussion

In our study in stable, minimally symptomatic, chronic HFrEF patients, repeatedly measured LV dimensions and left atrial dimensions, and E/A and E/E'-ratio were significantly associated with adverse cardiac events during a median follow-up of 2.3 years, independent of both baseline and repeated NT-proBNP measurements. Higher E/A-ratio and E/E'-ratio, both representing diastolic function of the heart, were the strongest predictors. Although repeated echocardiographic measurements were associated with cardiovascular outcome, they remained stable during follow-up and did not worsen as an adverse event approached. Thus, patients with adverse cardiac events had lower LVEF, larger LV dimensions and larger ratios throughout the entire follow-up period, but the temporal trajectories of these parameters did not show diverging slopes in patients with events vs those without events. This stability of the echo parameters was confirmed by the comparable HRs per SD increase found in the single measurement Cox models and the corresponding repeated measurements joint models, which further underscores the lack of incremental value of repeated echocardiograms compared to single echocardiographic assessment in this category of patients. Finally, we showed that repeated NT-proBNP measurements carry more prognostic information than repeated echo measurements do.

The current HF guideline does not recommend periodically repeating echoes in otherwise stable HF patients; reassessing myocardial structures and functions is only warranted when patients present with symptoms of worsening HF or experience any important cardiovascular event, prior to device implantations, and during exposure to cardiotoxic therapies[14]. However, this is primarily based on expert opinion. The results of our study are the first to explicitly substantiate that single echocardiographic assessment suffices in the context of prognosis. In our study we performed echocardiography each six months. Increasing the frequency of the echocardiographic assessments might improve the ability of echocardiography to reveal deterioration of myocardial function and structure prior to adverse events. However, the feasibility in clinical practice would be limited as this would significantly increase costs. Furthermore, subtle deterioration may not always be recognized as it cannot easily be distinguished from changes caused by normal intrapatient variability and intra- and inter-observer variability[15].

Finally, prolonging follow-up may also reveal incremental value of repeated echocardiography.

The prognostic value of baseline echo parameters, independent of baseline NT-proBNP, has been reported earlier in HF patients. In a prospective study by Hinderliter et al. among 211 chronic HFrEF patients, baseline LVEF, LV end-diastolic volume and LV volume index were significantly associated with all-cause mortality during a median of 4 years follow-up[4]. Furthermore, a systematic review of risk prediction models in patients with heart failure showed that baseline LVEF was often incorporated in the final models together with (NTpro)BNP levels, indicating LVEF carried additional prognostic value[16]. In our analysis, LVEF went from being not predictive to predictive for single vs repeated measures (the latter being assessed by means of joint modeling). This was most likely caused by the fact that repeated measurements entail higher statistical power. Both analyses show similar point estimates, but in the repeated measurement analysis a much higher number of LVEF measurements were taken into account, leading to narrower 95%CIs and thus a statistically significant finding.

Although it is thus clear that echocardiographic parameters carry prognostic value independent of NT-proBNP, in our study we extend the evidence by comparing effect sizes; by standardizing the variables we demonstrate that the association of NT-proBNP with adverse outcomes in CHF is much stronger than that of echocardiographic parameters. Several prior studies have already established that single baseline measurements of NT-proBNP are strongly associated with adverse outcomes in CHF[17-19]. In addition, an earlier study from our research group that used joint modeling has demonstrated that repeatedly measured NT-proBNP can identify high-risk periods in the Bio-SHiFT patients[8]. In that study, patients in whom the endpoint occurred had on average not only a higher baseline level of NT-proBNP, but also showed a significant rise of NT-proBNP levels as the endpoint approached. Similar results were reported in a study by Miller et al. among 190 NYHA III and IV CHF patients during a follow-up of 2 years, in whom BNP was measured every 3 months[20]. Altogether, previous studies taken together with the current study, suggest that for individualized assessment of CHF status and treatment, repeated NT-proBNP measurements are more useful than repeated echocardiographic assessments.

Limitations

A first limitation is that the treating physicians were not blinded to the echocardiograms. Due to ethical considerations this was not possible. A second limitation is that the number of endpoints in the current investigation is limited, and consequently the number of variables that could be entered in the models. Although residual confounding will surely be present, all our models were corrected for either baseline level or repeated measurements of NT-proBNP, which is a strong predictor of outcomes in chronic heart failure[21,22]. In addition, the longitudinal trajectories were also corrected for any differences in baseline characteristics between those who reached the endpoint and those who did not. The limited number of endpoints has also precluded us from investigating whether there would be differences in the results as a function of etiology of heart failure. Thirdly, we did not examine global longitudinal strain nor the grade of diastolic function, although these parameters could potentially predict clinical outcome. However, the use of GLS is limited in clinical practice and it is often not measured by default. As for grade of diastolic function, it is known that in practice classification of diastolic function is not always straightforward. When attempting to perform such a classification using the ASE guidelines[23], we were able to assign the grade in 61.3% of the echocardiograms only. This precluded performing a robust analysis. Finally, compared to previous CHF cohorts and also to the full Bio-SHiFT cohort, the patients in the our current echo study were relatively young and the proportion of HF patients in NYHA class I and II was high, which may have obscured an association that would be present in 'sicker' patients.

Conclusions

Although individual trajectories of echocardiographic parameters were associated with cardiovascular outcome independent of NT-proBNP levels, the parameters remained stable during 2.3 years follow-up and did not worsen as an adverse event approached. It thus seems that, in such a timeframe, routine frequent monitoring of systolic or diastolic function with echocardiography does not carry incremental prognostic information over a single measurement in a, largely, minimally symptomatic and relatively young patient group.

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Chapter 10

Longitudinal patterns of NT-proBNP, troponin T and CRP in relation to the dynamics of echocardiographic parameters in heart failure patients

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Abstract

Aims

To further elucidate the nature of the association between NT-proBNP, hs-TnT, CRP, and clinical outcome, we examined the relationship between serial simultaneous measurements of echocardiographic parameters and these biomarkers in chronic heart failure (CHF) patients.

Methods and results

In 117 CHF patients with ejection fraction ≤50%, NT-proBNP, hs-TnT, and CRP were measured simultaneously with echocardiographic evaluation at six-month intervals until the end of 30 months-follow-up or until an adverse clinical event occurred. Linear mixed effects (LME) models were used for data-analysis. Median follow-up was 2.2 years [IQR: 1.5-2.6]. We performed up to 6 follow-up evaluations with 55% of patients having at least 3 evaluations performed. A model containing all three biomarkers revealed that doubling of NT-proBNP was associated with a decrease in left ventricular EF by 1.83 (95% CI:-2.63;-1.03)%, p<0.0001; relative increase in mitral E/e' ratio by 12 (6,18) %, p<0.0001); relative increase in mitral E/A ratio by 16 (9;23)%, p<0.0001; decrease in TAPSE by 0.66 (-1.27;-0.05) mm, p=0.03; rise in TRPG by 2.74 (1.43;4.05) mmHg, p=0.001 and increase in left ventricular and atrial dimensions, p<0.05. Hs-TnT and CRP showed significant associations with some echocardiographic parameters after adjustment for clinical covariates, but after adjustment for the other biomarkers the associations were not significant.

Conclusion

Serum NT-proBNP independently reflects changes in echocardiographic parameters of systolic function, left ventricular filling pressures, estimated pulmonary pressure and chamber dimensions. Our results support further studies on NT-proBNP as a surrogate marker for hemodynamic congestion and herewith support its potential value for therapy guidance.

Introduction

Blood biomarkers form an easily accessible tool for diagnosis of heart failure (HF) and for evaluation of patient prognosis. Previous studies have demonstrated that the temporal evolutions of biomarkers of wall stress, myocyte damage and inflammation such as NT-proBNP, high sensitivity cardiac troponin T (hs-TnT) and high sensitivity C-reactive protein (CRP) predict clinical outcome among patients with heart failure[1-5]. Such temporal biomarker evolutions enable personalized prognostication, which is an important advantage compared to mere single, 'baseline' biomarker measurements[1].

Linking temporal biomarker evolutions to changes within myocardial structure and function could provide additional insights into the mechanisms that underlie above-described associations between blood biomarkers and clinical outcome. Previous studies on the relationship between NT-proBNP or cardiac troponin and echocardiographic evaluation have mostly performed the biomarker measurements at a single moment in time[6-10]. Studies that have performed longitudinal biomarker assessment in this context are scarce and involved only one follow-up echocardiographic evaluation[4] or used simple mathematical approaches (such as rise above the reference threshold or absolute change of both the biomarker and echocardiographic parameters)[11,12]. Moreover, these studies have focused on the evaluation of particular echocardiographic indices (left ventricular ejection fraction [LVEF] or LVEF in combination with LV end-diastolic diameter [LVdD] and LV end-systolic diameter [LVsD]). However, no studies have been conducted so far that analyzed the temporal pattern of both biomarkers and echocardiographic measures based on more than two simultaneous measurements and that used a comprehensive mathematical approach.

With this study we aimed to further explore the pathophysiology behind the associations of NT-proBNP, hs-TnT and CRP with patient prognosis. For this purpose, we have performed a prospective investigation that simultaneously assesses longitudinal patterns of these biomarkers and of multiple echocardiographic parameters in heart failure patients. We hypothesized that temporal patterns of blood biomarkers and echocardiographic parameters are correlated.

Methods

Study design

The design of the Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT) study has been presented in detail previously[1]. Bio-SHiFT is a prospective, observational study of stable patients with chronic heart failure (CHF) conducted in Erasmus MC, Rotterdam, the Netherlands and Noordwest Ziekenhuisgroep, Alkmaar, the Netherlands. Recruitment of patients was performed during their regular outpatient visits. Detailed inclusion and exclusion criteria are presented in Figure S10.1. Patients with a diagnosis of heart failure according to the European Society of Cardiology Guidelines set at least 3 months ago and in stable clinical condition during their regular outpatient visits were eligible for inclusion in the study[13]. All participants provided written informed consent. The study was approved by the medical ethics committee of the Erasmus MC, Rotterdam, the Netherlands and was conducted in accordance with the Declaration of Helsinki. The study is registered in ClinicalTrials.gov, number NCT01851538.

At baseline, patients were evaluated by trained research physicians, who interviewed the patients and performed physical examination. History of chronic kidney disease (CKD) was defined as glomerular filtration rate <60 ml/min/1.73 m^2. Blood samples were drawn. After inclusion, follow-up visits were scheduled every three months (a window of +/- one month was allowed), with a maximum of 10 follow-up visits. At each follow-up visit, a short medical evaluation was performed, blood samples were drawn, and occurrence of adverse cardiovascular events and medication changes since the previous visit were recorded. A total of 398 patients were included in Bio-SHiFT from August 2011 to January 2018. In the repeated echo sub-study, we included 106 patients. The echo sub-study was only performed at Erasmus MC, and consisted of pre-scheduled, repeated echocardiograms performed in the context of the study, every six months during follow-up, additional to the tri-monthly blood sampling in Bio-SHiFT. All the patients included into the Bio-SHiFT study were eligible for the echo substudy. For the current analysis, we enriched this set of 106 patients with 11 Bio-SHiFT patients that had repeated echocardiograms performed according to the same echo-protocol at Erasmus MC in the context of their usual care during the study period.

The final cohort subjected to this analysis (N=117 pts) comprised patients with EF ≤ 50%. Patients continued to undergo routine follow-up by their treating physicians independently of the study visits.

Biomarker measurement

All the samples were processed within 2 hours after blood collection. Sample transportation and storage were performed at a temperature of -80°C. NT-proBNP, hs-TnT and CRP were measured batch-wise using the methods described in the supplemental text (Text S10.1) and the results of the measurements were not available to the treating physicians. Therefore, participation in the study had no influence on patient care.

Echocardiographic assessment

All echocardiograms were performed and stored in the echo core lab of Erasmus MC. LVEF (3D triplane method), LVdD and LVsD, end-systolic left atrial diameter (LAsD) and tricuspid regurgitation peak systolic gradient (TRPG) were measured using specialized TOMTEC software (Unterschleissheim, Germany) . Inferior vena cava collapse and valvular function were assessed. Analysis was performed with the use of Philips Xcellera version R4.1 (Philips medical systems, the Netherlands). The early (E) and atrial (A) inflow velocities as well as early diastolic mitral annular velocity (e') were measured in order to calculate E/A and E/e'. For the e', we used the average of the lateral and medial e' when available; however, if only one of the two was available, this value was used. Each of the echocardiographic parameters was measured by one sole observer; thus eliminating the issue of inter-observer variability. All echocardiographic measurements were performed blinded to biomarker and clinical event data.

Statistical analysis

Distributions of continuous variables were tested for normality using the Shapiro-Wilk test. Normally distributed continuous variables are presented as mean and standard deviation, non-normally distributed continuous variables as median and interquartile range (IQR). Categorical data are displayed as count and percentage. In case of skewed distributions, continuous variables were logarithmically transformed (log base 2) for further analyses.

The evolution of echo parameters and biomarkers over time was estimated by using linear mixed (longitudinal) models with time entered as the independent variable and biomarkers or echo parameters entered consecutively as the dependent variable, assuming random intercepts and when appropriate also random slopes.

To evaluate the associations between repeated echocardiographic and biomarker measurements we again used linear-mixed-effects models. Blood biomarkers were consecutively entered as the independent variable, and echocardiographic parameters were consecutively entered as the dependent variable. First, analyses were adjusted for age and gender (Model I). Second, other potential confounders, selected based on the existing literature, were also entered into the model (atrial fibrillation, CKD , diabetes mellitus, SBP, body mass index (BMI), ischemic heart disease) – Model II. Third, every biomarker was adjusted for the other 2 biomarkers, age and gender – Model III.

All analyses were performed with R v. 3.4.4 Statistical Software using package 'nlme'. All tests were two-tailed and p-values <0.05 were considered statistically significant. An extended description of the statistical analysis can be found in the Supplementary data (Text S10.1).

Results

Baseline characteristics and follow-up visits

In total, 117 patients were evaluated. Baseline characteristics are presented in Table 10.1. The median follow-up was 803 (IQR: 532-966) days. Patients had up to 6 simultaneous echocardiographic and biochemical evaluations performed with 55% of patients having at least 3 evaluations. Mean age of the patients was 58±11 years, 80% were male and 76% in NYHA class I or II. The final echo substudy cohort comprised younger patients with higher prevalence of NYHA class I and II compared to the rest of study participants. Based on first LVEF available, 1(0.9%) HF patient (with EF=50%) presented with preserved ejection fraction (HF-PEF,≥50%), 11(9.4%) with mid-range ejection fraction (HF-mrEF,40%-49%) and 105 (89.7%) with reduced

ejection fraction (HF-REF,<40%) when classified according to the current guidelines[14]. In 246 out of 286 measurements, both lateral and medial e' were available; in 39, only medial e'; and in 1,only lateral e'. Mean LVEF was 27±9%. Our study population showed a tendency towards increased chamber diameters: LVdD (67±10mm), LVsD (59±11 mm), LAsD (44±9 mm). Mean TAPSE was reduced (17±5mm). TRPG above 36 mmHg was observed in 9(8%) patients at baseline. Baseline biomarker levels were as follows: NT-proBNP 118 (33-222)pmol/l, hs-TnT 15 (9-30)ng/l, CRP 1.7 (0.9-4.5)mg/l.

Temporal evolutions of blood biomarkers and echocardiographic parameters

In the total study population, the echocardiographic and biochemical parameters remained relatively stable over the course of the follow-up. We could not demonstrate any significant associations between time and most echocardiographic parameters (with the exception of E/A, p=0.04) or the biomarkers (NT-proBNP, hs-TnT, with the exception of CRP, p=0.0001) (Figure 10.1, Figure S10.2 for individual trajectories).

Table 10.1 Baseline characteristics of the study population.

Baseline characteristics (N=117)	
Demographics	
Age (years)	58±11
Male gender	93 (80%)
Caucasian ethnicity	105 (91%)
Clinical characteristics	
BMI (kg/m2)	27 (24–31)
Systolic blood pressure (mmHg)	110±18
Diastolic blood pressure (mmHg)	70 (60–79)
Pulse (beat/min)	67(60–72)
Features of HF	
Duration of HF at inclusion (years)	5 (2–10)
NYHA class III or IV	16 (14%)
Etiology of HF	
Ischemic heart disease	47 (40%)
Hypertension	0
Secondary to valvular disease	3 (3%)
Cardiomyopathy	52 (44%)
Dilated	41 (35%)
Hypertrophic	4 (3%)
Non-compaction	4 (3%)
Unclassified	3 (3%)

Table 10.1 (continued)

Baseline characteristics (N=117)	
Other	8 (7%)
Unknown	7 (6%)
Echocardiographic findings	
LVEF (%)	27±9
TAPSE (mm)	17±5
LVdD (mm)	67±10
LVsD (mm)	59±11
LAsD (mm)	44±9
TRPG (mmHg)	28±13
Biomarker concentrations	
NT-proBNP (pmol/l)	118 (33–222)
hs-TnT (ng/l)	15 (9–30)
CRP (mg/l)	1.7 (0.9–4.5)
Medical history	
Myocardial infarction	45 (38%)
Percutaneous coronary intervention	41 (35%)
Coronary artery bypass grafting	10 (9%)
Atrial fibrillation	32 (27%)
Pacemaker	41 (35%)
Implantable cardioverter-defibrillator	89 (76%)
Cardiac resynchronization therapy	36 (31%)
Stroke (CVA/TIA)	13 (11%)
Chronic kidney disease	48 (41%)
Diabetes mellitus	31 (26%)
Known hypercholesterolemia	49 (42%)
Hypertension	49 (42%)
Intoxications	
Alcohol intake (>1 unit/d)	21 (18%)
Smoking	
Current	14 (12%)
Former (>30 days)	62 (54%)
Medication use	
Beta-blocker	113 (97%)
ACE-I	88 (75%)
Angiotensin receptor blocker	28 (24%)
Aldosterone antagonist	75 (64%)
Diuretic	109 (93%)
Digoxin	61 (52%)

Categorical variables are expressed as count (percentage). Values of continuous variables are expressed as mean ± standard deviation or as median (interquartile range) in case of skewed distribution. Abbreviations: ACE-I, angiotensin-converting enzyme inhibitor; BMI, body mass index; EF, ejection fraction; LAsD, left atrial systolic diameter; LVdD, LV end-diastolic diameter. LVsD – LV end-systolic diameter; TAPSE, tricuspid annular plane systolic excursion; TIA, transient ischemic attack; TRPG, tricuspid regurgitation peak gradient.

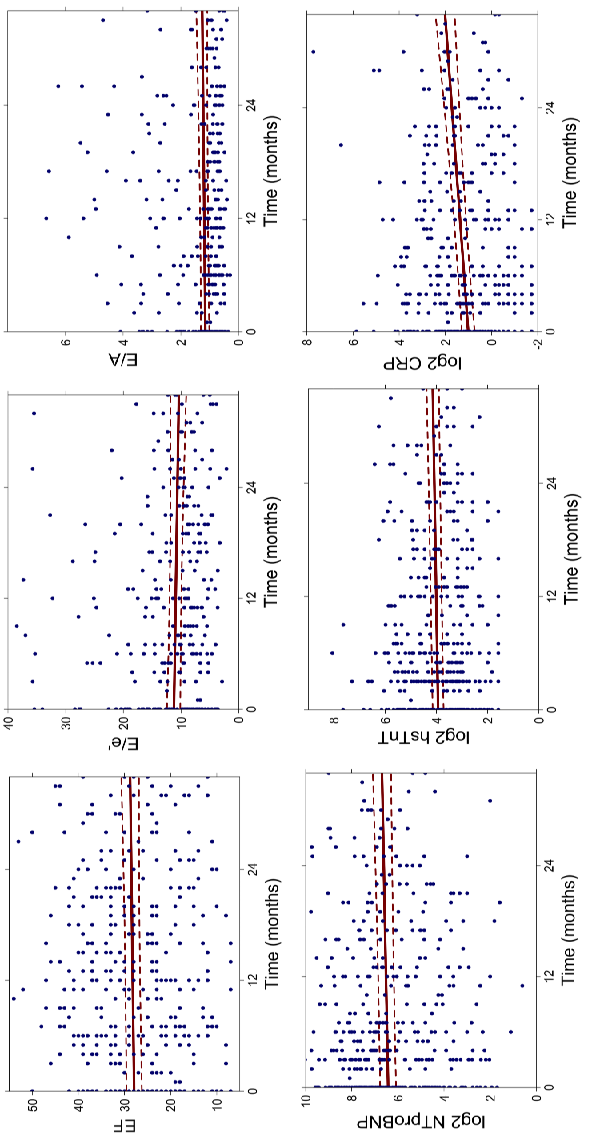

Figure 10.1 **Temporal evolution of biomarkers and echocardiographic parameters.** Solid lines represent the estimate of the mean level over time from an unadjusted linear mixed effects model and dashed lines represent 95%CI. A – peak late filling velocity. E – peak early filling velocity, e' – early diastolic mitral annular velocity, EF – left ventricular ejection fraction.

Associations between serial biomarker measurements and serial echocardiography

We found no significant multicollinearity in our models (all VIFs<2.5). Because of their skewed distributions, biomarkers, E/e' and E/A entered into the models were $\log_2$ transformed. NT-proBNP was significantly associated with all investigated echocardiographic parameters after adjustment for age and sex (Model I, Table S10.1), and almost all of these associations were significant after adjustment for clinical variables (Model II, Table 10.2) as well as for hs-TnT and CRP (Model III, Table 10.2). Doubling of NT-proBNP was associated with a decrease by 2.21 (95% confidence interval, CI:-2.85;-1.57)% in LVEF p<0.0001 in model II (clinically adjusted), an increase by 3.03(0.09;0.20) mmHg in pulmonary pressure estimated by TRPG, p<0.0001 and a decrease in deceleration time (DT) by 12(-10;-5)ms, p=0.0004. Doubling of NT-proBNP was associated with a 13(8;18)% relative increase in E/e' ratio, p<0.0001 and 16(10;21)% relative increase in E/A ratio, p<0.0001. A positive association with cardiac chamber dimensions including LVdD, LVsD and LAsD was observed (Table 10.2). For hs-TnT, upon univariable analysis we found significant associations with several echocardiographic parameters (Model I, Table S10.1), most of which were significant after adjustment for clinical variables (Model II, Table 10.2), a decrease by 2.35(-3.46;-1.25)% in LVEF, p<0.0001, a rise of E/e' ratio by 12(4;19)%, p=0.03, a rise of E/A ratio by 19(7,32)%, p=0.0007, an increase by 1.59(0.74;2.45) mm in LAsD, increase in TRPG by 3.95(1,52;5,27) mmHg, p=0.005 per doubling of hs-TnT serum concentration, as well as positive associations with chamber dimensions. However, these associations were not significant after accounting for the other two biomarkers (Model III, Table 10.2). Doubling of CRP was associated with an increase in E equal to 2.72(0.68;4.76) cm/s, p=0.009, an increase in E/A ratio by 6(1,13)%, p=0.02 and a rise in TRPG by 1.16(0.18,2.14) mmHg, p=0.02 in the clinically adjusted model (Model II, Table 10.2), but again no significant associations were found in the combined 3-biomarker model (Model III, Table 10.2). Associations between repeatedly measured biomarkers (NT-proBNP, CRP, hs-TnT) and repeatedly measured echocardiographic parameters (EF, E/e' ratio, E/A ratio) are presented in Figure 10.2. Subgroup analyses of patients with AF and those with sinus rhythm are presented in the supplementary data (Tables S10.2 and S10.3).

Table 10.2 Associations between repeatedly measured echocardiographic parameters and repeatedly measured biomarkers.

Echo parameter	NT-proBNP (pmol/l)				hs-TnT (ng/L)				CRP (mg/L)			
	Model II		Model III		Model II		Model III		Model II		Model III	
	B (95%CI)	p	B (95%CI)	p	B (95%CI)	p	B (95%CI)	p	B (95%CI)	p	B (95%CI)	p
LVEF (%)	-2.21	<0.0001	-1.83	<0.0001	-2.35	<0.0001	-0.83	0.25	-0.14	0.27	0.48	0.08
	(-2.85,-1.57)		(-2.63;-1.03)		(-3.46;-1.25)		(-2.13;0.45)		(-0.72;0.43)		(-2.21;0.39)	
E (cm/s)	6.18	<0.0001	6.66	<0.0001	3.32	0.11	-2.76	0.26	2.72	0.009	1.67	0.12
	(3.78;8.57)		(3.83;9.49)		(-0.79;7.43)		(-7.58;2.07)		(0.68;4.76)		(-0.43;3.78)	
E/e' *	0.18	<0.0001	0.17	<0.0001	0.16	0.03	-0.01	0.92	0.03	0.35	-0.02	0.92
	(0.11;0.24)		(0.09;0.24)		(0.06;0.27)		(-0.15;0.14)**		(-0.03;0.09)		(-0.05;0.05)	
E/A *	0.21	<0.0001	0.22	<0.0001	0.25	0.0007	0.0002	0.65	0.09	0.02	0.01	0.65
	(0.14;0.28)		(0.13;0.30)		(0.10;0.40)**		(-0.14;0.14)		(-0.02;0.18)**		(-0.05;0.08)	
A (cm/s)	-1.41	0.14	-2.49	0.02	-0.38	0.81	0.88	0.66	0.48	0.51	1.22	0.23
	(-3.29;0.48)		(-4.64;-0.34)		(-3.54;2.78)		(-3.04;4.81)		(-0.96;1.91)		(-0.80;3.24)	
DT (ms)	-11.76	0.0004	-12.01	0.002	-10.36	0.06	-12.00	0.98	2.78	0.009	1.67	0.12
	(-19.19;-5.33)		(-19.60;-4.42)		(-21.08;0.36)		(-19.59;-4.42)		(0.76;4.80)		(-0.42;3.78)	
LVdD (mm)	1.43	<0.0001	1.14	0.002	1.40	0.01	-0.39	0.52	-0.87	0.76	2.38	0.41
	(0.83;2.05)		(0.43;1.85)		(0.33;2.39)		(-0.83;1.62)		(6.54;4.79)		(-3.39;8.15)	
LVsD (mm)	2.24	<0.0001	1.99	<0.0001	1.70	0.008	-0.14	0.85	0.20	0.36	-0.10	0.66
	(1.51;2.97)		(1.14;2.85)		(0.44;2.97)		(-1.62;1.34)		(-0.23;0.64)		(-0.59;0.38)	
LAsD (mm)	1.54	<0.0001	1.35	<0.0001	1.59	0.003	0.86	0.09	0.41	0.13	-0.009	0.83
	(1.04;2.04)		(0.77;1.94)		(0.74;2.45)		(-0.23;1.86)		(-0.13;0.97)		(-0.60;0.58)	
TAPSE (mm)	-0.45	0.06	-0.66	0.03	-0.58	0.15	-0.49	0.35	0.01	0.94	0.33	0.19
	(-0.93;0.03)		(-1.27;-0.05)		(-1.37;0.22)		(-2.55;0.56)		(-0.46;0.49)		(-0.27;0.82)	
TRPG	3.03	<0.0001	2.74	0.001	3.95	0.005	0.51	0.59	1.16	0.02	0.26	0.59
(mmHg)	(1.95;4.11)		(1.43;4.05)		(1.52;5.27)		(-1.71;2.74)		(0.18;2.14)		(-1.71;2.73)	

Biomarkers in all the models are $\log_2$ transformed – beta (B) values express a mean change in parameter per doubling of biomarker value. Example based on Model II: doubling of NTproBNP reflects a mean decrease in LVEF(%) by 2.21. * For the analysis of E/e' and E/A, echocardiographic parameters were $\log_2$ transformed in order to satisfy the requirements for linear regression. In this case the mean change in echocardiographic parameter is calculated by multiplying by $2^{\wedge beta.}$ Example: doubling of NTproBNP reflects a mean rise in E/e' by $2^{\wedge 0.18} = 1.13$, which means a mean 13 % rise in E/e'. ** Model includes random slope for biomarker. 95% CI–95% confidence interval, A–peak late filling velocity, DT–E wave deceleration time, e'–early diastolic mitral annular velocity, E–peak early filling velocity, LVEF–left ventricular ejection fraction, LAsD–left atrial systolic diameter, LVdD–LV end-diastolic diameter, LVsD–LV end-systolic diameter, TAPSE–tricuspid annular plane systolic excursion, TRPG–tricuspid regurgitation peak gradient.

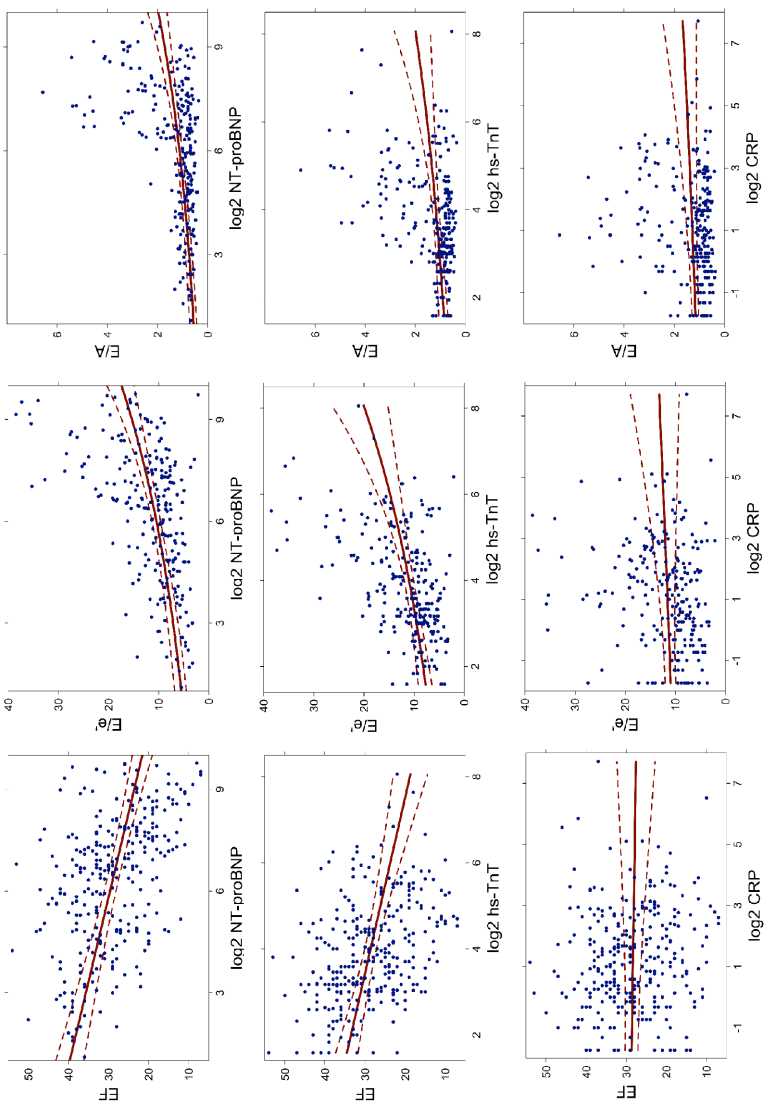

Figure 10.2 Associations between repeatedly measured biomarkers and repeatedly measured echocardiographic parameters. Solid lines represent the estimates of the mean echo parameter values over the range of the biomarkers, from an unadjusted linear mixed effects model and dashed lines represent 95%CI. A – peak late filling velocity. E – peak early filling velocity, e' – early diastolic mitral annular velocity, EF – left ventricular ejection fraction.

Discussion

In this prospective, observational study we demonstrate a significant and independent association between the individual, temporal evolution of NT-proBNP and the individual, temporal evolution of multiple echocardiographic parameters in CHF patients. These parameters include measures of systolic and diastolic function as well as LV geometry, and the associations persist over 2.2 years of follow-up. Conversely, we found that although hs-TnT correlates with most of these echocardiographic parameters and that CRP is associated with some diastolic function parameters, neither of them show significant associations if evaluated in a model together with NT-proBNP.

Both single and serial NT-proBNP measurements have been shown to carry prognostic information in HF patients in the chronic as well as acute setting[5,15-18]. Moreover, single, 'baseline' measurements of NT-proBNP are known to be correlated with echocardiographic parameters(8). Studies that have related longitudinal NT-proBNP measurements to echocardiography are fewer in number and, with the exception of the study by Sato et al.[11], have usually performed no more than two echocardiographic evaluations[4,12]. To the best of our knowledge, so far no study has investigated the temporal relationship between an elaborate set of echocardiographic indices and NT-proBNP, with both being repeatedly measured; thus, our study provides a unique opportunity to obtain additional insights into their relation, and to herewith gain a better understanding of the mechanisms underlying the predictive value of NT-proBNP as reported by other researchers[1]. We have observed that NT-proBNP is correlated with major functional parameters such as LVEF, diastolic function indices (E, E/e', E/A, and DT), chamber dimensions (LVdD, LVsD, LAsD) as well as an estimate of pulmonary pressure (TRPG). Our findings suggest that NT-proBNP may reflect both progression of the disease in the long term (e.g. gradual LVEF deterioration, increasing dilation of the heart chambers) as well as significant hemodynamic changes, which may occur more rapidly (e.g. elevation in filling pressures).

Among the parameters significantly associated with NT-proBNP, the correlation with parameters that reflect left ventricular filling pressure (LVFP) is of special clinical importance. These include parameters that have been reported as independent predictors of LVFP (E/e', E/A) or are components of models predicting

LVFP (TRPG)[19]. Clinically silent increased left ventricular filling pressure, which is transmitted back to the pulmonary vessels is termed "hemodynamic" congestion and is present already days or weeks prior to clinically overt congestion[20-23]. It has been proven that therapies based on the monitoring of hemodynamic congestion reduce HF hospitalizations[24]. The results of our study provide an additional argument in favor of the use of NT-proBNP as a surrogate marker of rising LVFP within the frames of natriuretic peptide guided therapy, for which sufficient evidence is currently still lacking[14]. Previous studies examining its efficacy in HF patients have yielded conflicting results[25]. However, these natriuretic peptide guided therapy trials mostly used predefined time points to measure natriuretic peptides, as well as predefined target levels. Such a predefined strategy does not account for individual temporal patterns of natriuretic peptides, and may hamper their potential use for therapy guidance. Trials addressing personalized strategies should be considered.

Contrary to NT-proBNP, our results suggest that neither hs-TnT nor CRP are independent biomarkers of the dynamics of LV dysfunction or remodelling. Pathophysiologically, inflammation, oxidative stress and neurohormonal activation can induce myocyte injury, reflected by troponin release[26]. Troponin is also reported to be a marker of unfavourable outcome[27,28]. However, troponin is influenced by other factors such as comorbidities including renal dysfunction, coronary artery disease and diurnal variation[29]. Our results are also consistent with other reports such as the MOCA study[30].

CRP reflects distinct inflammatory pathways within the diseased myocardium. It has previously been shown to be a marker of poor outcome and the magnitude of its reduction following anti-inflammatory treatment has been shown predictive of clinical outcome[31,32]. Its serum level depends on many factors[26] and its association with poor outcome may therefore represent the effect of comorbidities such as coronary artery disease, diabetes mellitus and CKD. Cytokines and cytokine receptors such as IL-6 and TNF-R1 may carry potential for detecting inflammatory processes in the myocardium[33], and investigation of these cytokines in relation to echocardiographic parameters merits consideration.

Some aspects of this study warrant consideration. We examined 3 biomarkers, and in such settings correction for multiple testing may be considered. However, our

study was not data driven but hypothesis driven; the biomarkers we investigated were implicated in heart failure by earlier studies and correcting for multiple testing could result in failure to recognize potentially interesting associations. All patients come from one center and with 117 patients, the sample size is limited. Fewer echocardiographic evaluations were included in the analysis than was anticipated, due to a combination of factors, including sporadic unavailability of echocardiographers at the time of the study visit, which led to rescheduling of the echocardiographic evaluation and its subsequent exclusion from the current analysis due to different timing than blood sampling; and, in some cases, early occurrence of the primary endpoint. The majority of patients were in NYHA class I or II. Moreover, all patients in our study had CHF with reduced LVEF, and results can therefore not be extrapolated to CHF patients with preserved LVEF[34]. Furthermore, given the observational nature of the study, recommendations on NT-proBNP guided therapy cannot be inferred. It should be taken into account, that our analysis explores temporal correlations, and does not infer that one biomarker or echocardiographic characteristic precedes or causes changes in the other. Nevertheless, this is the first study using longitudinal and simultaneous measurements of biomarkers and echo parameters, and taking into account their full temporal patterns by applying linear mixed effects modelling, which increases the accurateness of the analysis compared to the methods applied before and allows us to draw conclusions on the temporal correlations between biomarkers and echocardiographic measures[12].

In conclusion, serum NT-proBNP reflects the dynamics of change in echocardiographic parameters, including ejection fraction, indicators of ventricular filling pressures (E/A and E/e') and TRPG as an estimate of pulmonary pressure. Conversely, Hs-TnT and CRP do not seem to independently reflect the dynamics of functional and morphological parameters of the myocardium.

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Chapter 11

Longitudinally measured fibrinolysis factors are strong predictors of clinical outcome in patients with chronic heart failure - the Bio-SHiFT study

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Abstract

Objective

To investigate whether longitudinally measured fibrinolysis factors are associated with cardiac events in patients with chronic heart failure (CHF).

Methods

A median of 9 (IQR 5-10) serial, tri-monthly blood samples per patient were prospectively collected in 263 CHF patients during a median follow-up of 2.2 (IQR 1.4-2.5) years. 70 patients (cases) reached the composite endpoint of cardiac death, heart failure hospitalization, LVAD or heart transplantation. From all longitudinal samples, we selected baseline samples in all patients and the last two samples before the event in cases or the last sample available in event-free patients. Herein, we measured PAI-1, tPA, uPA, and suPAR. Associations between temporal biomarker patterns during follow-up and the cardiac event were investigated using a joint model.

Results

Cases were on average older and showed higher NYHA class than those who remained event-free. They also had lower blood pressures, and were more likely to have diabetes, renal failure and atrial fibrillation. Longitudinally measured PAI-1, uPA and suPAR were independently associated with adverse cardiac events after correction for clinical characteristics (hazard ratio (95% confidence interval) per SD increase of 2.09(1.28 -3.45) for PAI-1, 1.91(1.18–3.24) for uPA, and 3.96(2.48–6.63) for suPAR. Serial measurements of tPA were not significantly associated with the event after correction for multiple testing.

Conclusion

Longitudinally measured PAI-1, uPA and suPAR are strongly associated with adverse cardiac events during the course of CHF. If future research confirms our results, these fibrinolytic factors may carry potential for improved, and personalized, heart failure surveillance and treatment monitoring.

Introduction

It is well known that patients with chronic heart failure (CHF) carry increased risk of thromboembolic events. Previous research has linked CHF to all three categories of Virchow's triad (hypercoagulability of the blood, endothelial injury of the vessel walls, and stasis of the blood) that contribute to thrombophilia[1-3]. The degree to which thrombotic risk is increased has been shown to be related to HF severity and occurrence of adverse cardiac events in CHF patients[4,5]. In parallel, levels of fibrinolytic factors also seem to be related to adverse cardiac events in this patient population. Specifically, existing studies have associated single 'baseline' measurements of fibrinolytic factors with adverse clinical outcomes in CHF patients[6-8]. However, as CHF is heterogeneous and plasma levels of coagulation and fibrinolysis factors are variable, distinguishing high-risk individual patients using a single measurement is challenging. We hypothesize that by using repeated measurements of these factors, individual patterns can be identified which contribute to personalized risk assessment.

In the current study comprising 263 CHF patients, blood sampling was performed every three months. We set out to longitudinally measure the plasma concentration of four fibrinolysis factors in the baseline samples of all patients, and the last two available samples before the study endpoint, or the last available measurement in patients that remained endpoint-free. The fibrinolysis factors were: Plasminogen Activator Inhibitor 1 (PAI-1), tissue-type Plasminogen Activator (tPA), urokinase-type Plasminogen Activator (uPA), and soluble urokinase Plasminogen Activator surface Receptor (suPAR). Using these serial measurements, we aimed to investigate whether upregulation of the fibrinolytic cascade during the course of CHF is associated with occurrence of adverse cardiac events.

Methods

Details of the Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT) study design have been published previously[9]. Briefly, Bio-SHiFT is a prospective, observational study of stable patients with CHF conducted in the Erasmus MC, Rotterdam, and in the Northwest clinics, Alkmaar, the

Netherlands. Patients were recruited during their regular outpatient visits and were eligible when: (1) aged 18 years or above, (2) diagnosed with CHF according to the guidelines of the European Society of Cardiology three or more months before inclusion[10], and (3) if they were stable, i.e. they had not been hospitalized for HF in the past three months. After inclusion, study follow-up visits were scheduled every three months (+/- one month). At baseline and at each follow-up visit, a short medical evaluation was performed, blood samples were collected, and occurrence of adverse cardiac events since the last visit was recorded. During the study, the routine outpatient follow-up and treatment according to the ESC guidelines by the treating physician continued. These physicians were not aware of the results of the biomarker measurements during regular outpatient follow-up, as biomarker measurements were performed batch-wise after study follow-up was completed. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrial.gov (NCT01851538). Written informed consent was obtained from all patients. The current investigation comprised 263 CHF patients enrolled during the first inclusion period (October 2011 until June 2013).

Clinical study endpoints

The primary endpoint comprised the composite of cardiac death, cardiac transplantation, left ventricular assist device (LVAD) implantation and hospitalization for the management of acute or worsened HF, whichever occurred first in time. All events were adjudicated by a clinical event committee blinded to biomarker results, after reviewing corresponding hospital records and discharge letters.

Cardiac death was defined as death from myocardial infraction or other ischemic heart disease (*International Classification of Disease, 10th Revision*: I20-I25*)*, death from other heart disease including HF (I30-I45 and I47-I52), sudden cardiac death (I46), sudden death undefined (R96), or unwitnessed or ill-described death (R98, R99). Hospitalization for acute or worsened CHF was primarily based on exacerbation of HF symptoms requiring hospitalization. On top of this, a combination of 2 of the following was required: BNP or NT-proBNP >3x upper limit of normal; signs of worsening HF, such as pulmonary rales; raised jugular venous pressure or peripheral edema; increased dose or intravenous administration of diuretics; or administration of positive inotropic agents.

Blood sampling and laboratory analysis

Blood samples were collected at baseline and at each study follow-up visit, and were processed and plasma was stored at -80 degree Celsius within two hours after collection. For this analysis, we selected all baseline samples for the current investigation, and additionally the last two samples drawn before occurrence of the composite endpoint, or the last sample available for patients in whom the primary endpoint did not occur during follow-up. The samples were shipped to Uppsala, Sweden for a batchwise analysis using the Cardiovascular panel III (Olink Proteomics AB, Uppsala, Sweden). This assay is based on proximity extension assay technology[11]. In brief, the assay uses two oligonucleotide-labeled antibodies to bind to their respective target proteins in the sample. When the two antibodies are in close proximity, a new PCR target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using standard real-time PCR. Four internal controls and two external controls were included in each assay. The raw Cq values were normalized for variation between and within runs and converted into Normalized Protein Expression Units (NPX). Thus, these NPX values are relative values that result from the PCR. They are expressed on a Log2 scale, on which a one unit higher NPX value represents a doubling of the measured protein concentration. This arbitrary unit can be used for relative quantification of proteins and for comparing the fold changes between groups.

For the current investigation, we focused on the four fibrinolysis factors from the Cardiovascular panel III: PAI-1, tPA, uPA, and suPAR.

Statistical analysis

Distributions of continuous variables were visually inspected for normality. Normally distributed continuous variables are presented as mean ± standard deviation (SD), and non-normally distributed variables as median and interquartile range (IQR). Differences between groups were tested using Student t-tests (normally distributed continuous variables) or Mann Whitney tests (non-normally distributed continuous variables). Categorical variables are presented as numbers and percentages and differences between groups were tested with chi-square tests. We used linear mixed-effects (LME) models, to study the relationship between baseline characteristics and temporal biomarker patterns. The selected

biomarkers were modelled as dependent variables, whereas follow-up time and the baseline characteristics were considered independent variables. We also used LME models to plot the average temporal pattern of each fibrinolysis factor for patients with and without the composite endpoint during study follow-up and for investigating the associations between the individual biomarkers.

To estimate the associations between patient-specific repeated biomarker measurements and the hazard of the composite endpoint, we applied Bayesian semi-parametric joint modeling. These joint models combine linear mixed effect models for the temporal evolution of the repeated measurements, with Cox proportional hazard models for the time-to-event data, herewith enabling simultaneous estimation of the LME- and Cox model parameters. Unadjusted hazard ratio (HR) estimates for each biomarker were obtained, as well as estimates adjusted for age, gender, platelet inhibitor and anticoagulants use and all baseline variables that significantly differed between incident cases and patients that remained event-free during follow-up, namely NYHA-class, use of diuretics, diabetes, atrial fibrillation, systolic blood pressure, and renal function ('clinical' model). Finally, in a third model ('biomarker' model) we investigated if the associations between the fibrinolysis factors and clinical outcomes were affected by baseline levels of established heart failure biomarkers N-terminal pro-Brain Natriuretic peptide (NT-proBNP), high-sensitivity troponin T and C-reactive protein (CRP), as well as baseline levels of cathepsin D. Cathepsin D is also a marker on the Cardiovascular panel III and known to influence fibrinolysis[12]. Data on all variables were complete, except for systolic blood pressure which was missing in 5% of the patients. These missing values were imputed using the patients' clinical and outcome data. For the biomarker analysis, we used the Z-score (i.e. the standardized form) of the $\log_2$-transformed biomarkers to allow for direct comparisons of the different fibrinolytic factors. Correspondingly, results are given as hazard ratios (HR) and 95% confidence intervals (CI) per SD increase of the biomarker's level.

To evaluate model performance, we estimated the level of the fibrinolytic factors at the time of event or censoring with the joint model, and used this to compute the c-index and the Likelihood Ratio (LR) Chi-square test.

All tests were two-tailed. We corrected for multiple testing using Bonferonni correction. Consequently, the corrected significance level was set at $p<0.013$. We used the conventional $p<0.05$ threshold to establish the significance of the associations between baseline patient characteristics and biomarker levels, as well as for the relation between baseline characteristics and the occurrence of the primary endpoint. All analyses were performed with R statistical software using package 'nlme'[13] and 'JMbayes'[14].

Results

Baseline characteristics are presented in Table 11.1. The median (IQR) age of the patients was 68 (59-76) years, 72% were men, and 74% were in NYHA class <III at baseline. Most patients had CHF with reduced ejection fraction (95%) and ischemic heart disease was the most common etiology (45%).

During a median (IQR) follow-up of 2.2 (1.4-2.5) years, 70 (26.6%) patients reached the primary, composite endpoint. Nine patients died of cardiovascular causes, 3 patients underwent heart transplantation, 2 patients underwent LVAD placement, and 56 patients were hospitalized for acute or worsened HF. Patients who reached the endpoint during follow-up were significantly older and more often in a higher NYHA-class, than patients who did not reach the endpoint. They also had lower blood pressures, higher baseline NT pro-BNP, higher prevalence of diuretics use, and were more likely to have diabetes, renal failure, and atrial fibrillation.

Association between baseline characteristics and longitudinal biomarker measurements

Table 11.2 shows the associations between the baseline characteristics and the longitudinally measured fibrinolytic factors. Except for uPA, all fibrinolytic factors were significantly associated with age (beta per 10 years (95% CI) for age; PAI-1: -0.21 (-0.29, -0.13); tPA: 0.10 (0.03, 0.18); suPAR: 0.19 (0.13, 0.24)), while no statistically significant association with gender was found. The presence of known atrial fibrillation at baseline was associated with substantial increase of the fibrinolytic factors, except for PAI-1. Deterioration of kidney function was

associated with lower levels of PAI-1 and higher levels of uPA and suPAR, while tPA was on average not affected.

Table 11.1 Baseline characteristics.

		Composite endpoint reached		
Variable	**Overall**	**No**	**yes**	**p**
N (%)	263 (100)	193 (73.4)	70 (26.6)	
Demographics				
Age, years (median, IQR)	68 (59, 76)	67 (58, 75)	72 (61, 80)	0.02
Men, n (%)	189 (72)	136 (70.5)	53 (75.7)	0.50
Clinical characteristics, mean (SD)				
BMI, kg/m2	27.5 (4.9)	27.5 (4.7)	27.3 (5.4)	0.77
Heart rate, bpm	67 (12)	67 (11)	69 (13)	0.27
Systolic blood pressure, mmHg	122 (20)	124 (21)	117 (17)	0.02
Diastolic blood pressure, mmHg	72 (11)	73 (11)	70 (10)	0.06
Features of heart failure				
NYHA class ≤II	194 (73.8)	155 (80.3)	39 (55.7)	<0.001
NYHA class ≥III	69 (26.2)	38 (19.7)	31 (44.3)	<0.001
HF-rEF, n (%)	250 (95.1)	184 (95.3)	66 (94.3)	0.75
HF-pEF, n (%)	13 (4.9)	9 (4.7)	4 (5.7)	0.75
LVEF, % (mean (SD))	31 (11)	31 (11)	28 (11)	0.12
NT pro-BNP, pmol/L (median (IQR))	137.3 (51.8, 271.6)	95.3 (32.8, 205.9)	282.4 (177.4, 503.3)	<0.001
Etiology of heart failure, n (%)				
Ischemic	117 (44.5)	81 (42.0)	36 (51.4)	0.24
Hypertension	34 (12.9)	24 (12.4)	10 (14.3)	0.69
Secondary to valvular disease	12 (4.5)	7 (3.6)	5 (7.1)	0.23
Cardiomyopathy	68 (25.9)	53 (27.5)	15 (21.4)	0.32
Other or unknown	32 (12.2)	28 (14.5)	4 (5.7)	0.06
Medical history, n (%)				
Prior MI	95 (36.3)	63 (32.8)	32 (45.7)	0.08
Prior PCI	82 (31.4)	55 (28.8)	27 (38.6)	0.17
Prior CABG	43 (16.5)	30 (15.6)	13 (18.8)	0.67
Atrial fibrillation	105 (40.4)	69 (36.3)	36 (51.4)	0.04
Diabetes	81 (30.8)	49 (25.4)	32 (45.7)	0.003
Renal failure	136 (52.3)	89 (46.4)	47 (69.1)	0.002
COPD	31 (11.9)	19 (9.9)	12 (17.4)	0.15
Medication use, n (%)				
Beta-blocker	236 (89.7)	175 (90.7)	61 (87.1)	0.55
ACE-Inhibitor	174 (66.2)	128 (66.3)	46 (65.7)	1.000
ARB	77 (29.3)	59 (30.6)	18 (25.7)	0.54
Loop diuretics	236 (89.7)	168 (87.0)	68 (97.1)	0.03
Aldosterone antagonist	179 (68.1)	126 (65.3)	53 (75.7)	0.15

N: number; IQR: interquartile ranges; SD: standard deviation; BMI: body mass index; kg: kilogram; m2: squared meter; bpm: beats per minute; mmHg millimeter of mercury; NYHA: New York Heart Association; HF-rEF: heart failure with reduced ejection fraction; HF-pEF: heart failure with preserved ejection fraction; LVEF: left ventricular ejection fraction; NT pro-BNP: N-terminal pro-brain natriuretic peptide; pmol: picomole; L: liter; MI: myocardial infarction; PCI: percutaneous coronary intervention; CABG: coronary artery bypass grafting; COPD: chronic obstructive pulmonary disease; ACE: angiotensin converting enzyme; ARB: angiotensin II receptor blockers.

Table 11.2 Association between patient characteristics and repeatedly measured fibrinolytic biomarkers during follow-up.

Dependent variable	PAI-1		tPA		uPA		suPAR	
Independent variable	**Beta (95% CI)**	**p-value**	**Beta (95% CI)**	**p-value**	**Beta (95% CI)**	**p-value**	**Beta (95% CI)**	**p-value**
Age per 10 years	-0.21 (-0.29, -0.13)	<0.001	0.10 (0.03, 0.18)	0.006	0.03 (-0.01, 0.07)	0.10	0.19 (0.13, 0.24)	<0.001
Male gender	0.09 (-0.14, 0.32)	0.43	0.14 (-0.07, 0.34)	0.20	-0.05 (-0.15, 0.06)	0.39	-0.11 (-0.27, 0.05)	0.18
NYHA Class	-0.18 (-0.31, -0.04)	0.01	0.11 (-0.02, 0.23)	0.09	0.04 (-0.02, 0.10)	0.24	0.25 (0.16, 0.34)	<0.001
Diabetes mellitus	0.12 (-0.10, 0.34)	0.30	-0.08 (-0.28, 0.12)	0.42	0.10 (0.00, 0.19)	0.06	0.35 (0.21, 0.50)	<0.001
Atrial fibrillation	0.03 (-0.18, 0.24)	0.78	0.23 (0.04, 0.41)	0.02	0.23 (0.14, 0.32)	<0.001	0.33 (0.19, 0.47)	<0.001
SBP per 10 mmHg	-0.04 (-0.09, 0.01)	0.10	0.01 (-0.03, 0.06)	0.57	-0.04 (-0.06, -0.01)	0.001	-0.02 (-0.06, 0.01)	0.20
eGFR per 20ml/min/1.73m2	0.14 (0.05, 0.23)	0.004	-0.03 (-0.11, 0.06)	0.55	-0.06 (-0.10, -0.02)	0.004	-0.25 (-0.30, -0.19)	<0.001
NT-proBNP per doubling pmol/L	-0.10 (-0.15, -0.05)	<0.001	0.03 (-0.02, 0.08)	0.21	0.07 (0.05, 0.10)	<0.001	0.18 (0.15, 0.21)	<0.001

The effects of patients' baseline characteristics on temporal pattern of the biomarkers are given as Betas (95% confidence interval) per doubling of the level. Hence, a beta of 1 corresponds with a 2fold higher biomarker level. PAI-1: Plasminogen activator inhibitor 1; tPA: Tissue-type plasminogen activator; uPA: Urokinase-type plasminogen activator; suPAR: soluble urokinase plasminogen activator surface receptor; NYHA: New York Heart Association; SBP: Systolic blood pressure; eGFR: estimated glomerular filtration rate; NTproBNP: N-terminal prohormone of brain natriuretic peptide.

Association between the individual fibrinolysis factors

All fibrinolytic factors were significantly correlated with each other. In line with known physiological pathways, PAI-1 showed the strongest association with tPA, while uPA showed the strongest association with suPAR. An overview of all associations can be found in Supplementary Table S11.2.

Longitudinally measured biomarkers and study endpoints

Figure 11.1 and Supplementary Table S11.1 show the average levels of the fibrinolytic factors at baseline and at the last available measurement, both for patients that reached the endpoint and for patients that did not. In patients that remained endpoint-free, levels of PAI-1 and suPAR were similar at baseline and in the last samples. Conversely, in patients with cardiac events, these factors were significantly higher in the last samples drawn before the endpoint, compared to baseline. For tPA and uPA, levels became significantly lower over time in endpoint-free patients, while levels remained stable in patients that experienced adverse cardiac events.

Figure 11.2 shows the average trajectories for patients who experienced the endpoint and for endpoint-free patients. As the endpoint grew near, the levels of the fibrinolytic factors rose; although this was less outspoken for PAI. When assessed with joint modeling, the trajectories of all four investigated fibrinolytic factors were positively associated with the cardiac events. suPAR had the strongest association with the cardiac events with a HR (95%confidence interval) of 3.15 (2.30-4.48) per SD increase in the univariate analysis and 3.96 (2.48-6.63) in the adjusted ‘clinical’ model. Although its estimate was substantial, tPA was the only marker that did not reach a statistically significant effect after Bonferroni correction in both the crude (HR 1.97 (1.02-4.16)) and the clinical model (HR 3.00 (1.22-10.00)). In contrast, all the fibrinolytic factors were significantly associated with adverse cardiac events when adjusted for baseline levels of the commonly used cardiovascular biomarkers NT-proBNP, hsTnT, CRP as well as cathepsin D (Table 11.3). In addition, for each of the fibrinolytic factors, a model containing the fibrinolytic factor performed better than a model with only the established heart failure biomarkers. The performance of the models containing the different

fibrinolytic factors and that of the model containing only the established biomarkers can be found in Table 11.4.

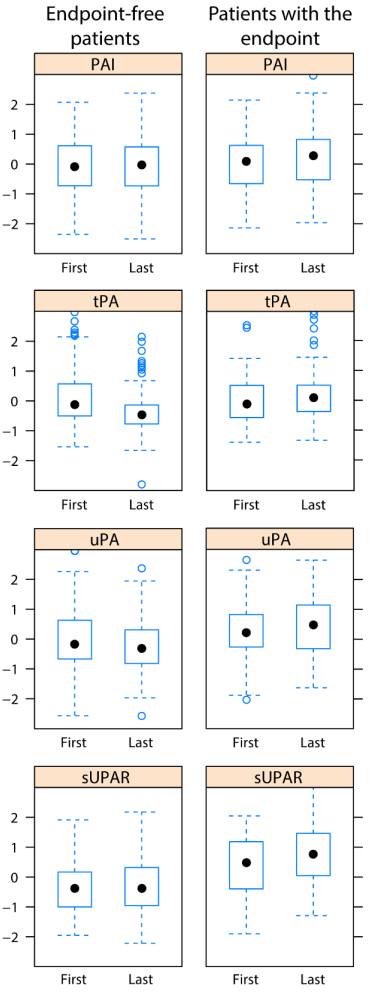

Figure 11.1 Average biomarker levels at the first and last measurement. The left boxplots show the average biomarker levels in cardiac event-free patients; the right boxplots show the levels in patient with an adverse cardiac event. Within each boxplot, the left box represents the average of the first 'baseline' measurement. The right box depicts the average of the last available measurement for each patient. The Y-axis shows the Z-score.

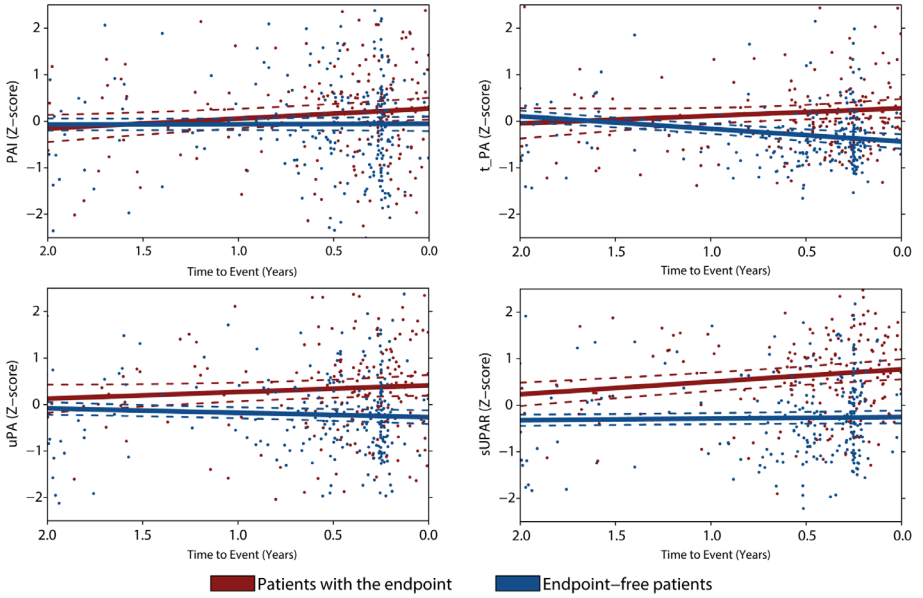

Figure 11.2 Average temporal pattern of the fibrinolytic biomarkers. The Y-axis shows the Z-score, where for example 0 and 1 represent the mean value and 1SD, respectively. The X-axis represents the time remaining to the primary endpoint (for patients who experienced adverse cardiac events) or time remaining to last sample moment (for patients who remained event-free). Of note is that 'time zero' is defined as the occurrence of the adverse cardiac event and is depicted on the right side of the x-axis, so that the average marker trajectory can be visualized as the event approaches. The continuous lines represent the average temporal pattern for patients with events (red) and event-free patients (blue). The corresponding dotted lines depict the 95% confidence intervals. Finally, each dot represents a single measurement.

Table 11.3 Assocation between biomarker patterns and the clinical endpoint.

	Crude		Clinical model		Biomarker model	
	HR (95%CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
PAI-1	1.47 (1.02 - 2.12)	0.042	2.05 (1.25 - 3.34)	0.004	1.73 (1.18 - 2.60)	0.005
tPA	1.97 (1.02 - 4.16)	0.044	2.68 (1.13 - 7.60)	0.024	2.26 (1.09 - 5.28)	0.030
uPA	2.27 (1.49 - 3.55)	<0.001	1.92 (1.16 - 3.23)	0.006	1.57 (0.98 - 2.63)	0.063
sUPAR	3.15 (2.30 - 4.48)	<0.001	3.92 (2.46 - 6.49)	<0.001	2.71 (1.73 - 4.49)	<0.001

Estimates are given as HR per increase of standard deviation of the NPX units. The clinical models are adjusted for age, gender, NYHA class at baseline, use of diuretics, diabetes status, atrial fibrillation, renal function and systolic blood pressure. The biomarker model was adjusted for baseline CRP, hsTnT, and NTproBNP. To correct for multiple testing, a p-value of <0.013 (Bonferroni correction: 0.05/4) was considered statistically significant. PAI-1: plasminogen activator inhibitor 1; tPA: tissue-type plasminogen activator; uPA: urokinase-type plasminogen activator; sUPAR: soluble urokinase plasminogen activator surface receptor.

Table 11.4 Performance of fibrinolysis biomarker models compared to traditional markers.

	C-index	LR χ^2	p-value
Established markers*	0.786	76.6	ref
Established markers plus PAI-1	0.805	87.81	0.009
Established markers plus tPA	0.871	145.6	<0.001
Established markers plus uPA	0.801	84.74	0.005
Established markers plus sUPAR	0.793	85.27	0.046

*NT-proBNP,HsTnT and CRP. Estimates are given for NT-proBNP,HsTnT and CRP, consecutively combined with one of the fibrinolysis factors. We used the levels at the time of event or censoring as estimated by the joint model to compute the performance measures. PAI-1: plasminogen activator inhibitor 1 ; tPA: tissue-type plasminogen activator; uPA: urokinase-type plasminogen activator; sUPAR: soluble urokinase plasminogen activator surface receptor.

Discussion

In this prospective study of 263 CHF patients who were followed for 2.2 years, those who experienced the endpoint had on average higher levels of longitudinally measured uPA and suPAR than patients who remained endpoint-free. Although longitudinally measured tPA was also positively associated with the endpoint, this association lost statistical significance after Bonferroni correction. PAI-1 only showed a statistically significant association with the endpoint after multivariable adjustment. When models were adjusted for baseline levels of NT-proBNP, hsTnT, CRP, and cathepsin D, all fibrinolytic factors models showed independent associations.

The findings from this investigation suggest that serial measurements of fibrinolytic factors can be used for identifying “high-risk” CHF patients. Recognizing and treating these patients more aggressively in an even earlier stage could prevent HF hospitalization. This is crucial, as mortality is known to be high after HF hospitalization[15,16]. Our study distinguishes itself from earlier studies investigating the role of fibrinolytic markers in CHF, as we have performed repeated blood sampling based on a pre-specified study protocol at fixed three-month intervals over the full course of follow-up, with up to 11 samples per patient. By selecting the baseline samples and the last two measurements prior to the composite endpoint or the last available measurement in those who remained endpoint-free, we made sure to select measurements that were close in time to the adverse cardiac endpoint. Herewith, we were able to take into account the change in

biomarker levels as adverse cardiac events approach[17], which has not been done previously.

The four fibrinolytic factors investigated in the current study are involved in the fibrinolytic cascade. Both tPA and uPA convert inactive plasminogen to active plasmin. Both activators have short half-lives in the circulation (4-8 minutes) as they are inhibited, especially by PAI-1. In order to prevent inhibition and to preserve their fibrinolytic activity, tPA and uPA can bind to several cell surface molecules, including uPAR[18]. uPAR itself is negatively regulated by uPA, which cleaves uPAR from the cell surface, turning it into suPAR. suPAR levels however, can also rise due to other processes, such as immune activation[19]. Given the interrelations between these fibrinolysis factors, one might hypothesize that any potential associations with clinical outcome in heart failure would show opposite directions for PAI-1 on the one hand and tPA, uPA and suPAR on the other hand, with the latter 3 factors showing protective effects. However, we observed that all factors were positively associated (although not all statistically significant) with the occurrence of the endpoint. A possible explanation might be that in the natural course of heart failure, all fibrinolysis factors in the cascade (both agonistic factors as well as antagonistic factors) are upregulated as a reaction to the prevailing thrombotic state, and that the degree of upregulation can be seen as an indicator of disease severity.

There are few reports on the prognostic value of the fibrinolytic markers in CHF patients. In a study by Jug et al. among 195 patients with CHF in NYHA class II and III, baseline antigen level of tPA above the cut-off of 10.2 microgram/liter was a strong and independent predictor of HF related deaths and hospitalizations in a multivariable Cox model (HR 2.70 (95%CI 1.23-5.36) during a median follow up of approximately 2 years.[6] We did not find a statistically significant association of tPA with our endpoint after Bonferroni correction. The differences in results might in part have resulted from differences in study population (the patients examined by Jug et al. showed worse functional status) or by the dichotomization of the biomarker. In contrast, in the same paper baseline antigen levels of PAI-1 were not significantly associated with adverse outcomes, while PAI-1 was a significant predictor in our study after corrections for clinical variables or commonly measured biomarkers. This is explained by the fact that we examined the full temporal trajectory of PAI-1. In accordance with Jug et al, we found no difference in baseline

levels of PAI; however over time, the levels of PAI-1 increased in those patients in whom the endpoint occurred, while remaining stable in endpoint-free patients. In a study among 370 HF patients with preserved ejection fraction, baseline antigen levels of tPA/PAI-1 complexes were significantly associated with cardiovascular mortality during 9.7 years follow-up[8]. Similar to our study, antigen tPA levels alone were not significantly associated with the endpoint while PAI-1 was more strongly associated with the endpoint in the multivariable analysis than in the crude analysis. This stronger association in the multivariable analysis might have been caused by the negative association we observed between PAI-1 on the one hand, and age and NYHA class on the other hand. As higher age and NYHA class are positively associated with cardiac events in CHF patients, adjusting for these variables will likely enlarge the HR of PAI-1. Finally, the prognostic value of suPAR levels was investigated in a study by Koller et al. They showed in two separate cohorts of 319 stable CHF patients and 346 hospitalized HF patients, that high baseline levels of suPAR were strongly and independently associated with both all-cause and cardiovascular mortality. Interestingly, the levels were on average higher in the hospitalized patients, giving rise to the hypothesis that suPAR rises towards hospitalization. The findings from our study confirm this hypothesis. In addition to these results, Koller et al. showed that adding suPAR levels to a multivariable Cox model including among others NTproBNP levels and NYHA class, significantly improved discrimination and reclassification, more so than ST2[7]. We now extend these finding by showing that suPAR is also a strongly associated with adverse cardiac events in CHF patients with a lower NYHA class (mostly I or II). This is an important finding because especially in these patients health benefit can be achieved by improved risk stratification.

Limitations

Our cohort consisted largely of patients with systolic dysfunction in NYHA I and II. Thus, generalizability could be compromised. However, for suPAR, the strongest predictor of cardiac events in our study, similar results have already been reported in two different cohorts using baseline measurements and consisting of HF patients with worse functional status[7]. A limitation of the study is that the proximity extension assay used for determining the fibrinolytic factors, only provides relative factor levels. These values can be used for comparing the differences between patients and changes of the levels within a patient, but do not quantify the factor concentrations in an absolute manner. This limits the possibilities for clinical

implementation and the comparability with previous and future research. Future research should focus on confirming our results using robust assays that allow for direct quantification of the fibrinolytic factors.

Conclusions

During the natural course of heart failure, a variety of biological mechanisms are activated, among which the fibrinolytic system. Altogether, our study suggests that in CHF patients, the degree of upregulation of the fibrinolytic cascade is associated with adverse events during follow-up. If future research confirms our results, these fibrinolytic factors may prove to carry potential for improved heart failure surveillance and may also prove useful for personalized treatment monitoring in CHF patients.

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Chapter 12

Circulating biomarkers of cell adhesion predict clinical outcome in patients with chronic heart failure

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Abstract

Cardiovascular inflammation and vascular endothelial dysfunction are involved in chronic heart failure (CHF), and cellular adhesion molecules are considered to play a key role in these mechanisms. We evaluated temporal patterns of 12 blood biomarkers of cell adhesion in patients with CHF. In 263 ambulant patients, serial, tri-monthly blood samples were collected during a median follow-up of 2.2 (1.4–2.5) years. The primary endpoint (PE) was a composite of cardiovascular mortality, HF hospitalization, heart transplantation and implantation of a left ventricular assist device and was reached in 70 patients. We selected the baseline blood samples in all patients, the two samples closest to a PE, or, for event-free patients, the last sample available. In these 567 samples, associations between biomarkers and PE were investigated by joint modelling. The median age was 68 (59–76) years, with 72% men and 74% New York Heart Association class I–II. Repeatedly measured levels of Complement component C1q receptor (C1qR), Cadherin 5 (CDH5), Chitinase-3-like protein 1 (CHI3L1), Ephrin type-B receptor 4 (EPHB4), Intercellular adhesion molecule-2 (ICAM-2) and Junctional adhesion molecule A (JAM-A) were independently associated with the PE. Their rates of change also predicted clinical outcome. Level of CHI3L1 was numerically the strongest predictor with a hazard ratio (HR) (95% confidence interval) of 2.27 (1.66–3.16) per SD difference in level, followed by JAM-A (2.10, 1.42–3.23) and C1qR (1.90, 1.36–2.72), adjusted for clinical characteristics. In conclusion, temporal patterns of C1qR, CDH5, CHI3L1, EPHB4, ICAM2 and JAM-A are strongly and independently associated with clinical outcome in CHF patients.

Introduction

In recent decades, chronic heart failure (CHF) has emerged as a complex syndrome that involves a broad array of biological pathways[1,2]. In this context, CHF has been associated with endothelial dysfunction and low-grade inflammation[3]. Moreover, the role of the immune system in the development and progression of CHF has received considerable attention in recent years[4]. An essential step in this process is the adherence of circulating mononuclear cells to the vascular endothelium through binding of cell adhesion molecules (CAMs) that are expressed on the surface of these mononuclear cells, or on the endothelial cells, or on both[5]. Binding of the mononuclear cells to the endothelium leads to extravasation of these cells into the involved tissue[5], promoting structural deterioration, which eventually contributes to reduced cardiac function. Interestingly, enhanced expression of CAMs has been found within the myocardial microvasculature of patients with severe CHF as compared to healthy subjects[6], providing further support that vascular inflammation might be involved in the propagation and progression of CHF.

Different classes of CAMs have been identified, and among them are selectins, integrins, cadherins and the immunoglobulin superfamily[7]. In addition, several other molecules are involved in the cell adhesion processes. In more detail, selectins such as platelet (P)-selectin (SELP) are involved in the adhesion of leucocytes to activated endothelium and are known for the typical “rolling” of leucocytes on the surface of the endothelium. Other selectins such as endothelial (E)-selectin (SELE) are involved in the cell extravasation process. Integrins mediate the leucocyte adherence to the vascular endothelium and other cell–cell interactions[8]. Cadherins are an important family of calcium dependent cell–cell adhesion molecules. In addition to their structural role, they have been implicated in the regulation of signalling events[7]. For example, cadherin 5 (CDH5) is a major cell–cell adhesion molecule that forms adherens junctions[9]. Lastly, the immunoglobulin superfamily comprises a diverse group of proteins including intracellular adhesion molecule-1 (ICAM-1), ICAM-2 and ICAM-3, vascular adhesion molecule-1 (VCAM-1), platelet endothelial cell adhesion molecule 1 (PECAM-1) and others, which are expressed on the surface of the endothelial cells and are known for firm adhesion of leucocytes and transendothelial migration[10].

Shedding of CAMs from the cell surface results in measurable levels in peripheral blood[11], which can reflect overexpression of their membrane-bound forms. Since CAMs may thus reflect processes involved in CHF, the association of these circulating biomarkers with clinical outcome provokes interest. Temporal patterns of biomarkers of cell adhesion in CHF, and their associations with an adverse disease course, have not yet been examined. Therefore, in this study, we investigated 12 cell adhesion-related biomarkers repeatedly measured with the Olink Multiplex panel, which contains 92 known human cardiovascular biomarkers that have previously been extensively investigated in the literature as well as exploratory candidates that are thought to carry potential as new biomarkers. Specifically, here, we examined biomarkers from this panel related to the above-described mechanisms (SELP, SELE, CDH5, ICAM-2, and PECAM-1) and other potentially interesting biomarkers related to cell adhesion processes (complement component C1q receptor (C1qR), chitinase-3-like protein 1 (CHI3L1), contactin-1 (CNTN1), ephrin type-B receptor 4 (EPHB4), epithelial cell adhesion molecule (Ep-CAM), integrin beta-2 (ITGB2), and junctional adhesion molecule A (JAM-A). The aim of the present study was to evaluate the association between temporal patterns of these biomarkers of cell adhesion and clinical outcomes in stable patients with CHF.

Methods

Patient selection

A total of 263 patients enrolled in the 'Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis' (Bio-SHiFT) study were included in the Netherlands. The Bio-SHiFT study is a prospective, observational cohort study of stable patients with CHF. Patients used for the current investigation were enrolled during the first study inclusion period from October 2011 until June 2013, while follow-up lasted until 2015. Patients were recruited during their regular outpatient clinic visit, in the Erasmus MC in Rotterdam or in the Northwest Clinics in Alkmaar. To be eligible for this study, CHF had to be diagnosed ≥3 months ago according to European Society of Cardiology guidelines[12,13]. Also, patients had to be ambulatory and stable, i.e., they should not have been hospitalized for HF in the past three months. The study

design of the Bio-SHiFT study (including detailed inclusion and exclusion criteria) has been described in detail previously[14,15]. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). Written informed consent was obtained from all patients.

Study procedures

All patients underwent standard care at the outpatient clinic by their treating physicians, who were blinded for biomarker results. Additionally, study follow-up visits were predefined and scheduled every 3 months (±1 month). At the moment of enrolment and at each study follow-up visit, a short medical evaluation was performed, blood samples were collected and occurrence of cardiovascular events since last study visit was recorded. Blood samples were processed and stored at −80°C within two hours after collection. As biomarkers were measured after completion of follow-up, this information did not lead to change of treatment strategies since treating physicians were unaware of the study results.

Study endpoints

The primary endpoint (PE) was a composite of cardiac death, heart transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. A clinical event committee, blinded for the biomarker results, reviewed hospital records and discharge letters and adjudicated the study endpoints[14,15].

Blood sample selection

In this first inclusion period of the Bio-SHiFT study, we collected a total of 1984 samples in 263 patients before occurrence of the PE or censoring (median of 9 (25th–75th percentile: 5–10) blood samples per patient). For reasons of efficiency, we made a selection from these samples: we selected all samples at enrolment, the last sample available in patients in whom the PE did not occur during follow-up, and the two samples available closest in time prior to the PE (which, by design, were 3 months apart). Previous investigations in this cohort have demonstrated that levels of several biomarker change in the months prior to the incident adverse event[14,15]. Thus, by selecting the last two samples prior to the endpoint, we aimed

to capture this change. In event-free patients however, our previous investigations showed stable biomarker levels, in which case one additional biomarker sample suffices. In total, this selection amounted to 567 samples for the current analysis.

Biomarker measurements

To investigate new biomarkers, the cardiovascular panel III of the Olink Multiplex platform (Olink Proteomics AB, Uppsala, Sweden) was used for a batch-wise analysis. This multiplexing assay is based on proximity extension assay technology[16]. The assay uses two oligonucleotide-labelled antibodies to bind to their respective target proteins in the sample. When the two antibodies are in close proximity, a new polymerase chain reaction target sequence is formed. The resulting sequence is detected and quantified using standard real-time PCR. The proteins/biomarkers are delivered in Normalized Protein Expression (NPX) Units, which are relative units that result from the polymerase chain reaction. The NPX units are expressed on a log2 scale where one unit higher NPX represents a doubling of the measured protein concentrations. This arbitrary unit can thus be used for relative quantification of proteins and comparing the fold changes between groups. In the 567 selected samples, we measured C1qR, CDH5, CHI3L1, CNTN1, EPHB4, Ep-CAM, ICAM2, ITGB2, JAM-A, PECAM-1, SELE and SELP. In Appendix A Table A1, an overview is given of the adhesion molecule biomarkers included in this study, including abbreviations, synonyms and function.
Additionally, in all patients, N-terminal pro–B-type natriuretic peptide (NT-proBNP) and high-sensitive troponin T (hsTnT) were measured using electro-chemiluminescence immunoassays (Elecsys 2010; Roche Diagnostics, Indianapolis, IN, USA) as described before[14].

Statistical analysis

Variables with a normal distribution are presented as the mean ± standard deviation (SD), whereas the median and 25th–75th percentile are used in case of non-normality. Differences between groups were tested with Student t-tests (for normally distributed variables) or with Mann Whitney tests (non-normally distributed variables). Categorical variables were presented as counts and percentages and differences between groups were tested with chi square tests. We used linear mixed effect models to plot the average temporal pattern of each

adhesion molecule biomarker for patients with and without a PE during study follow-up.

To estimate the associations between patient-specific repeated biomarker measurements and the hazard of the PE, we applied joint modelling (JM) analyses. JM combines linear mixed effect models for temporal evolution of the repeated measurements with time-to event relative risk models for the time-to-event data[17]. By using the JM technique, analyses inherently accounted for different follow-up durations between patients[18]. We studied the predictive value of biomarker levels, as well as their rates of change (i.e., the slopes of the longitudinal biomarker trajectories). The latter analysis is of particular interest in situations where, for example, at a specific time point two patients show similar marker levels, but differed in rate of change of the marker[19]. First, all JM analyses were performed univariably. Subsequently, we considered a 'clinical model' and an 'established biomarker model', to adjust for potential confounders. The clinical model was adjusted for age, gender, diabetes mellitus, atrial fibrillation, New York Heart Association (NYHA) class, use of diuretics and systolic blood pressure, while the established cardiac biomarker model was adjusted for NT-proBNP and hsTnT (measured at study enrolment). For all the JM analyses, we used the Z-score (i.e., the standardized form) of the log2-transformed biomarkers to allow for direct comparisons of different biomarkers. Results are given as hazard ratios (HR) with their 95% confidence intervals (CI) per SD change of the biomarker's level or slope.

We used the conventional $p<0.05$ threshold to conclude significance for the relation between patient characteristics and the occurrence of the PE during follow-up (Table 12.1). For the other analyses, we corrected for multiple testing using the Bonferonni correction ($n=12$), which resulted in a corrected significance level of $p<0.004$. Analyses were performed with SPSS Statistics 24 (IBM Inc., Chicago, IL, USA) and R Statistical Software using packages nlme[20] and JMbayes[17].

Table 12.1 Patients characteristics in relation to the occurrence of the primary endpoint (PE).

Variable	Total	PE Reached during follow-up		p-value
		Yes	No	
	263 (100)	70 (27)	193 (73)	
Demographics				
Age—years	68 (59–76)	72 (60–80)	67 (58–75)	0.021 *
Men	189 (72)	53 (76)	136 (71)	0.40
Clinical characteristics				
Body Mass Index—kg/m^2	26 (24–30)	27 (24–30)	26 (24–30)	0.80
Heart rate—beats/min	67 ± 12	69 ± 13	67 ± 11	0.22
Systolic blood pressure—mmHg	122 ± 20	117 ± 17	124 ± 21	0.020 *
Diastolic blood pressure—mmHg	72 ± 11	70 ± 10	73 ± 11	0.06
Features of heart failure				
Duration of HF—years	4.6 (1.7–9.9)	6.8 (2.8–12.5)	3.8 (1.1–8.2)	0.002 *
NYHA class III or IV	69 (26)	31 (44)	38 (20)	<0.001 *
HF with reduced ejection fraction	250 (95)	66 (94)	184 (95)	0.75
HF with preserved ejection fraction	13 (5)	4 (6)	9 (5)	
Left ventricular ejection fraction	31 ± 11	28 ± 11	31 ± 11	0.108
Established biomarkers				
NT-proBNP—pmol/L	137 (52–273)	282 (176–517)	95 (32–208)	<0.001 *
HsTnT—ng/L	18 (10–33)	32 (21–50)	14 (8–27)	<0.001 *
eGFR—ml/min per 1.73m^2	58 (43–76)	53 (40–73)	59 (44–77)	0.20
Aetiology of heart failure				
Ischemic	117 (45)	36 (51)	81 (42)	0.17
Hypertension	34 (13)	10 (14)	24 (12)	0.69
Secondary to valvular disease	12 (5)	5 (7)	7 (4)	0.31
Cardiomyopathy	68 (26)	15 (21)	53 (28)	0.32
Unknown or Others	32 (12)	4 (6)	28 (15)	
Medical history				
Prior myocardial infarction	96 (37)	32 (46)	64 (33)	0.060
Prior percutaneous coronary intervention	82 (31)	27 (39)	55 (29)	0.12
Prior Coronary artery bypass grafting	43 (16)	13 (19)	30 (16)	0.56
Prior CVA/TIA	42 (16)	15 (21)	27 (14)	0.15
Atrial fibrillation	106 (40)	36 (51)	70 (36)	0.027 *
Diabetes Mellitus	81 (31)	32 (46)	49 (25)	0.002 *
Hypercholesterolemia	96 (37)	30 (43)	66 (34)	0.20
Hypertension	120 (46)	38 (54)	82 (43)	0.090
COPD	31 (12)	12 (17)	19 (10)	0.11
Medication use				
Beta-blocker	236 (90)	61 (87)	175 (91)	0.40
ACE-I or ARB	245 (93)	63 (90)	182 (94)	0.22
Diuretics	237 (90)	68 (97)	169 (88)	0.021 *
Loop diuretics	236 (90)	68 (97)	168 (87)	0.017 *
Thiazides	7 (3)	3 (4)	4 (2)	0.39
Aldosterone antagonist	179 (68)	53 (76)	126 (65)	0.11

Table 12.1 (continued)

Variable	Total	PE Reached during follow-up		p-value
		Yes	No	
	263 (100)	70 (27)	193 (73)	
Biomarker level at baseline in arbitrary unit (NPX values)				
C1qR	8.88 (8.56–9.27)	9.16 (8.78–9.50)	8.78 (8.50–9.20)	<0.001 *
CDH5	2.29 (2.00–2.67)	2.36 (2.12–2.84)	2.27 (1.96–2.60)	0.010 *
CHI3L1	7.68 (6.88–8.39)	8.08 (7.53–8.72)	7.47 (6.68–8.20)	<0.001 *
CNTN1	2.01 (1.72–2.25)	2.00 (1.68–2.22)	2.01 (1.75–2.27)	0.58
EpCAM	5.11 (4.38–5.82)	4.91 (4.40–5.71)	5.18 (4.36–5.90)	0.41
EPHB4	1.35 (1.08–1.66)	1.55 (1.19–1.95)	1.31 (1.05–1.58)	<0.001 *
ICAM-2	4.20 (3.88–4.59)	4.35 (4.00–4.64)	4.18 (3.85–4.51)	0.061
ITGB2	4.65 (4.39–4.90)	4.64 (4.41–4.96)	4.67 (4.39–4.89)	0.86
JAM-A	5.22 (4.64–5.80)	5.41 (4.79–6.02)	5.08 (4.56–5.71)	0.024 *
PECAM-1	4.74 (4.36–5.17)	4.77 (4.36–5.39)	4.70 (4.35–5.10)	0.32
SELE	2.89 (2.46–3.28)	3.06 (2.51–3.32)	2.84 (2.45–3.28)	0.40
SELP	8.84 (8.46–9.38)	8.98 (8.54–9.58)	8.78 (8.42–9.28)	0.087

Variables with a normal distribution are presented as the mean ± SD, whereas non-normally distributed continuous variables are expressed as the median (25th–75th percentile). Categorical variables are expressed as counts (percentages). Missing values <5% if applicable, except for systolic blood pressure (5.3%). * p-value <0.05. ACE-I: angiotensin-converting enzyme inhibitors, ARB: angiotensin II receptor blockers, C1qR: complement component C1q receptor, CDH5: cadherin 5, CHI3L1: chitinase-3-like protein 1, CNTN1: contactin-1, COPD: chronic obstructive pulmonary disease, CVA: cerebrovascular accident, eGFR: estimated glomerular filtration rate, Ep-CAM: epithelial cell adhesion molecule, EPHB4: Ephrin type-B receptor 4, HF: heart failure, HsTnT: high-sensitive troponin T, ICAM-2: intercellular adhesion molecule-2, ITGB2: integrin beta-2, JAMA: junctional adhesion molecule A, NPX, Normalized Protein Expression, NT-proBNP: N-terminal pro–B-type natriuretic peptide, NYHA: New York Heart Association, PECAM-1: Platelet endothelial cell adhesion molecule 1, SELE: E-selectin, SELP: P-selectin and TIA: transitory ischemic attack.

Results

Baseline characteristics and study endpoints

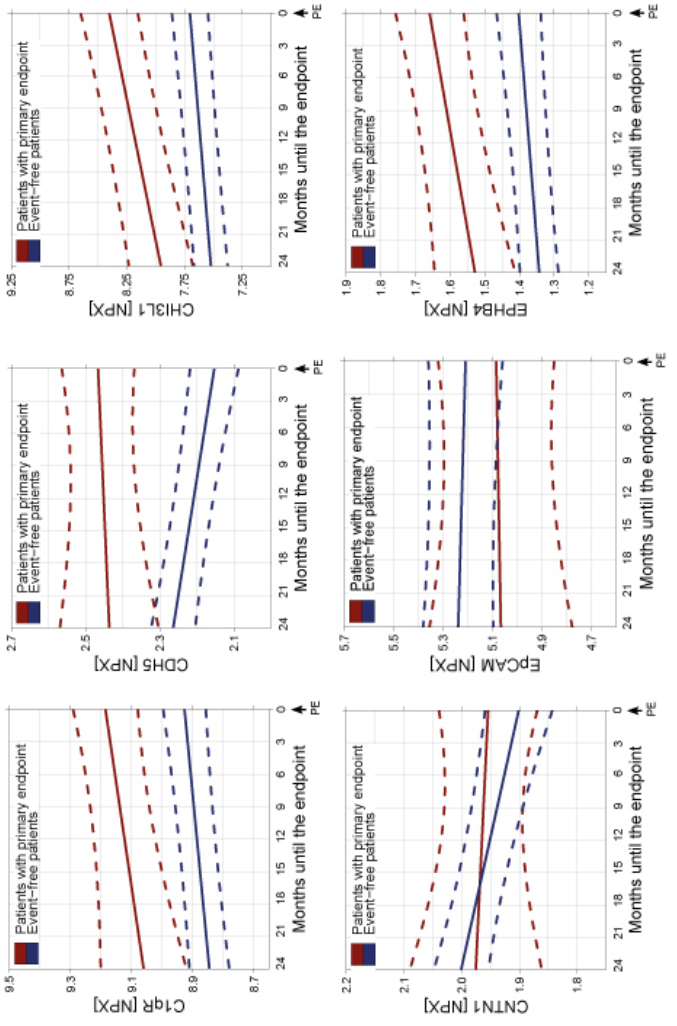
During a median (25th–75th percentile) follow-up of 2.2 (1.4–2.5) years, a total of 70 (27%) patients reached the PE: 56 patients were re-hospitalized for acute or worsened HF, three patients underwent heart transplantation, two patients underwent left ventricular assistant device implantation, and nine patients died of cardiovascular causes. Table 12.1 displays the patients' characteristics at enrolment and the differences in these characteristics between patients who reached the PE during follow-up and patients who did not. The median age was 68 (25th–75th

percentile: 59–76), years, with 72% men and 74% NYHA class I–II. The median duration of HF was 4.6 (1.7–9.9) years. Patients who reached the endpoint during follow-up were older and more often in a higher NYHA-class (III or IV), compared to patients who did not reach the PE. They also had a longer duration of HF, lower systolic blood pressures, higher levels of NT-proBNP and hsTNT, were more likely to have atrial fibrillation and diabetes mellitus, and had a higher prevalence of diuretics use. Baseline levels of C1qR, CDH5, CHI3L1, EPHB4 and JAM-A were significantly higher in patients who later experienced the endpoint compared to patients who remained event-free.

Temporal patterns of circulating biomarkers of cell adhesion in relation to study endpoints

Figure 12.1 depicts the average temporal evolutions of biomarkers of cell adhesion from twenty-four months before the PE or before last sample moment (for patients who remained event-free) onwards, based on linear mixed effect models. As the endpoint or last sample moment approached, biomarkers C1qR, CDH5, CHI3L1, EPHB4, ICAM-2 and JAM-A showed higher levels in patients who experienced the PE versus those who remained event-free. Some were already higher 24 months before the endpoint, while others were not but diverged as the end-point drew closer. On the other hand, CNTN1, EpCAM, ITGB2, PECAM-1, SELE and SELP did not show a clear difference between both groups.

Table 12.2 shows the associations of the repeatedly measured levels of biomarkers of cell adhesion with the PE based on JM analyses. C1qR showed the strongest association in univariate analysis with a HR of 2.22 (95% CI: 1.62–3.10) per SD change at any point in time during follow-up. After adjustment for clinical characteristics, CHI3L1 remained the strongest predictor of the PE, with a HR of 2.27 (95% CI: 1.66–3.16). CHI3L1 was followed by JAM-A (HR 2.10, 95% CI: 1.42-3.23) and C1qR (HR 1.90, 95% CI: 1.36–2.72). In addition, the risk estimates of CHI3L1 (HR 1.68, 95% CI: 1.23–2.35) and JAM-A (HR 1.75, 95% CI: 1.25–2.49) remained significant after adjustment for baseline established cardiac biomarkers NT-proBNP and hsTNT.


CHI3L1 [NPX]
EPHB4 [NPX]
CDH5 [NPX]
EPCAM [NPX]
C1qR [NPX]
CNTN1 [NPX]
Patients with primary endpoint
Event−free patients
Months until the endpoint
PE

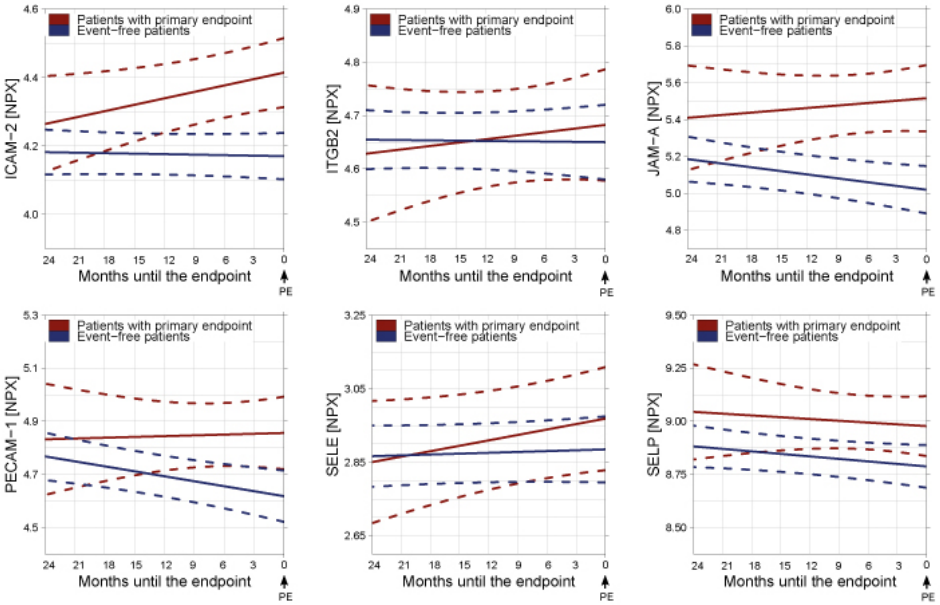


Figure 12.1 Average temporal patterns of adhesion molecule biomarkers during follow-up approaching the primary endpoint (PE) or last sample moment. *X*-axis: time remaining to the PE (for patients who experienced incident adverse events) or time remaining to last sample moment (for patients who remained event-free). Therefore, 'time zero' is defined as the occurrence of the endpoint or last sample moment and is depicted on the right side of the x-axis, so that the average marker trajectory can be visualized as the endpoint approaches. *Y*-axis: biomarker levels in arbitrary, relative units (Normalized Protein Expression, NPX). Solid red line: Average temporal pattern of biomarker levels in patients who reached the primary endpoint during follow-up. Solid blue line: Average temporal pattern of biomarker levels in patients who remained endpoint free (solid blue line). Dashed lines: 95% confidence interval. Abbreviations: Complement component C1q receptor: C1qR, Cadherin 5: CDH5, Chitinase-3-like protein 1: CHI3L1, CNTN1: Contactin-1, Ep-CAM: Epithelial cell adhesion molecule, EPHB4: Ephrin type-B receptor 4, , ICAM2: Intercellular adhesion molecule-2, ITGB2: Integrin beta-2, JAM-A: Junctional adhesion molecule A, NPX: Normalized Protein Expression, PE: primary endpoint, PECAM-1: Platelet endothelial cell adhesion molecule 1, SELE: E-selectin, and SELP: P-selectin.

Table 12.2 shows the associations of the repeatedly measured levels of biomarkers of cell adhesion with the PE based on JM analyses. C1qR showed the strongest association in univariate analysis with a HR of 2.22 (95% CI: 1.62–3.10) per SD change at any point in time during follow-up. After adjustment for clinical characteristics, CHI3L1 remained the strongest predictor of the PE, with a HR of 2.27 (95% CI: 1.66–3.16). CHI3L1 was followed by JAM-A (HR 2.10, 95% CI: 1.42-3.23) and C1qR (HR 1.90, 95% CI: 1.36–2.72). In addition, the risk estimates of CHI3L1 (HR 1.68, 95% CI: 1.23–2.35) and JAM-A (HR 1.75, 95% CI: 1.25–2.49) remained significant after adjustment for baseline established cardiac biomarkers NT-proBNP and hsTNT.

Table 12.2 Associations between the levels of biomarkers of cell adhesion and the primary endpoint.

	Crude Model		Clinical Model		Biomarker Model	
Biomarker	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
C1qR	2.22 (1.62–3.10)	<0.001*	1.90 (1.36–2.72)	<0.001*	1.47 (1.04–2.14)	0.028
CDH5	2.01 (1.47–2.77)	<0.001*	1.79 (1.30–2.50)	<0.001*	1.56 (1.14–2.14)	0.004
CHI3L1	2.11 (1.60–2.84)	<0.001*	2.27 (1.66–3.16)	<0.001*	1.68 (1.23–2.35)	0.002*
CNTN1	0.93 (0.66–1.32)	0.70	0.98 (0.67–1.45)	0.92	0.93 (0.66–1.31)	0.66
EpCAM	0.86 (0.66–1.11)	0.27	0.90 (0.67–1.20)	0.46	0.90 (0.69–1.17)	0.46
EPHB4	1.90 (1.48–2.44)	<0.001*	1.77 (1.35–2.33)	<0.001*	1.37 (1.03–1.80)	0.031
ICAM2	2.08 (1.51–2.94)	<0.001*	1.79 (1.29–2.53)	0.001*	1.53 (1.12–2.12)	0.005
ITGB2	1.07 (0.77–1.47)	0.70	0.95 (0.65–1.37)	0.77	1.04 (0.75–1.42)	0.83
JAM-A	1.86 (1.34–2.63)	<0.001*	2.10 (1.42–3.23)	<0.001*	1.75 (1.25–2.49)	0.001*
PECAM-1	1.39 (1.00–1.94)	0.050	1.60 (1.10–2.35)	0.013	1.47 (1.04–2.08)	0.031
SELE	1.11 (0.86–1.44)	0.43	1.07 (0.81–1.40)	0,66	1.11 (0.86–1.44)	0.43
SELP	1.34 (0.98–1.86)	0.071	1.45 (1.01–2.10)	0.044	1.49 (1.08–2.06)	0.018

Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per standard deviation change at any point in time during follow-up, which were estimated by joint modelling (JM) analysis. JM combines linear mixed effect (LME) models for the temporal evolution of the repeated measurements with Cox proportional hazard models for the time-to-event data. Thus, all available measurements are simultaneously taken into account in the current analyses (i.e., all baseline samples, the last sample available in patients in whom the PE did not occur during follow-up, and the two samples available closest in time prior to the primary endpoint). Crude model: Cox model unadjusted, LME model unadjusted; Clinical model: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline New York Heart Association class, diuretics, and systolic blood pressure; Established cardiac biomarker model: Cox and LME models adjusted for baseline NT-proBNP and hsTnT. Data for systolic blood pressure was missing in >5% of patients. Imputations were applied using the patients' clinical and outcome data. * *p*-value below the corrected significance level for multiple testing (*p*-value <0.004).

Apart from evaluating the predictive value of repeatedly assessed biomarker levels, we also evaluated their rates of change (i.e., the slopes of the longitudinal biomarker trajectories) and concurrent HRs. Although the trajectories plotted by

using linear mixed effect models (Figure 12.1) have already provided an impression of temporal evolution of biomarker level in those with and without incident PEs, evaluating slope by means of the JM provides the possibility to evaluate instantaneous slope, which may render additional insights. In these analyses, the same biomarkers remained significant predictors of the PE, i.e., CDH5, CD93, CHI3L1, EPHB4, ICAM-2 and JAM-A, even after adjusting for clinical factors (Table 12.3). JAM-A showed numerically the strongest association with the PE with a HR of 1.64 (95% CI: 1.23–2.24) per 0.1SD change of the annual slope, followed by CHI3L1 (HR 1.58, 95% CI: 1.36–1.93) and CDH5 (HR 1.47, 95% CI: 1.17–2.00).

Table 12.3 Associations between the slope of biomarkers of cell adhesion and the primary endpoint.

	Crude Model		Clinical Model		Biomarker Model	
Biomarker	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
C1qR	1.34 (1.16–1.56)	<0.001*	1.43 (1.13–1.92)	0.002*	1.12 (1.02–1.24)	0.019
CDH5	1.36 (1.18–1.60)	<0.001*	1.47 (1.17–2.00)	<0.001*	1.16 (1.07–1.27)	<0.001*
CHI3L1	1.41 (1.29–1.57)	<0.001*	1.58 (1.36–1.93)	<0.001*	1.27 (1.18–1.39)	<0.001*
CNTN1	1.04 (0.94–1.17)	0.45	1.04 (0.92–1.18)	0.53	1.06 (0.98–1.15)	0,13
EpCAM	1.01 (0.88–1.16)	0.92	1.01 (0.88–1.17)	0.88	1.01 (0.92–1.11)	0.83
EPHB4	1.33 (1.19–1.51)	<0.001*	1.34 (1.15–1.68)	<0.001*	1.14 (1.04–1.25)	0.005
ICAM2	1.32 (1.22–1.45)	<0.001*	1.44 (1.27–1.72)	<0.001*	1.22 (1.15–1.31)	<0.001*
ITGB2	1.07 (0.94–1.21)	0.32	0.99 (0.83–1.16)	0.90	1.05 (0.97–1.15)	0.23
JAM-A	1.34 (1.12–1.62)	0.002*	1.64 (1.23–2.24)	0.001*	1.10 (0.99–1.24)	0.085
PECAM-1	1.15 (0.98–1.40)	0.088	1.09 (0.86–1.72)	0.80	1.06 (0.97–1.18)	0.21
SELE	1.21 (1.05–1.41)	0.015	1.19 (0.99–1.41)	0.060	1.10 (0.96–1.23)	0.15
SELP	1.29 (1.13–1.49)	0.020	1.45 (1.22–1.84)	<0.001*	1.12 (0.94–1.27)	0.15

Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 0.1 standard deviation of the annual slope at any point in time during follow-up, which were estimated by joint modelling (JM) analysis. JM combines linear mixed effect (LME) models for the temporal evolution of the repeated measurements with Cox proportional hazard models for the time-to-event data. Thus, all available measurements are simultaneously taken into account in the current analyses (i.e., all baseline samples, the last sample available in patients in whom the PE did not occur during follow-up, and the two samples available closest in time prior to the primary endpoint). Crude model: Cox model unadjusted, LME model unadjusted; Clinical model: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline New York Heart Association class, diuretics and systolic blood pressure; Established cardiac biomarker model: Cox and LME models adjusted for baseline NT-proBNP and hsTnT. Data for systolic blood pressure was missing in >5% of patients. Imputations were applied using the patients' clinical and outcome data. * p-value below the corrected significance level for multiple testing (p-value <0.004).

Discussion

In the present study, we found that biomarkers of cell adhesion C1qR, CDH5, CHI3L1, EPHB4, ICAM-2 and JAM-A were associated with clinical outcomes in 263

stable patients with CHF. At baseline, levels of biomarkers C1qR, CDH5, CHI3L1, EPHB4 and JAM-A were higher in patients who later experienced the PE compared to patients who remained event-free. Furthermore, the average biomarker evolutions over time of these markers, and additionally of ICAM-2, showed higher levels as the PE approached. Even more important, repeatedly measured levels of these biomarkers of cell adhesion were independently associated with the PE. Even adjusted for clinical factors, biomarkers of cell adhesion served as predictors of clinical adverse events.

Recent studies suggest a pivotal role of CAMs in the processes of HF. Until now, however, research on CAMs in relation to adverse clinical outcomes in patients with CHF is limited. Previous studies have mostly described the value of single measurements of adhesion molecules (e.g., at admission) for prognosis, and studies were relatively small. Our study, which was based on repeated measurements, demonstrates a promising role for several adhesion biomarkers for individual prognostication in CHF patients temporal patterns shortly before an adverse event occurs have not yet been investigated in detail previously, while this might be a crucial time window for therapeutic interventions.

In our study, CHI3L1 was the biomarker whose association with the PE was numerically the strongest after adjustment for clinical factors. CHI3L1 is a glycoprotein secreted in vitro by cells such as activated macrophages and neutrophils in different tissues with inflammation. Studies on patients with acute myocardial infarction, stable coronary artery disease, atrial fibrillation and CHF have demonstrated elevated levels of CHI3L1 compared with healthy controls[21]. Moreover, several studies have previously examined CHI3L1 in relation to clinical outcome in CHF; but repeated measurements were never used. Some of these studies showed that CHI3L1 is associated with all-cause mortality[22] and that it is able to detect patients at high risk for adverse outcomes as well[23,24]. Other studies failed to demonstrate such associations. Rathcke at al. examined CHI3L1 levels in patients with CHF and in age-matched controls without cardiovascular disease[25]. They found higher levels of CHI3L1 at baseline in patients with CHF, but these levels did not predict cardiovascular events or overall mortality. Mathiasen et al.[21] suggested that, most likely, elevated levels of CHI3L1 in CHF patients are explained by the presence of concomitant diseases. CHF is a complex disorder, often complicated by other comorbidities in which CHI3L1 is known to be elevated, such

as arrhythmias, renal dysfunction, diabetes mellitus and hypertension. These concomitant diseases could thus possibly explain the differences in CHI3L1 levels when compared to healthy individuals. Conversely, in our study, we not only adjusted for age, but also for clinical factors, and still we found an association between CHI3L1 and clinical adverse events.

The barrier formed by endothelial cells allows regulated passage of immune cells in the normal state and during inflammatory conditions. This passage is mediated through junctional molecules, such as ICAM-2, CDH5, JAMA, and PECAM-1[26,27]. ICAM-2 participates in the docking of leukocytes to the endothelium, and is likely to be relevant for leukocyte diapedesis[28]. For example, former research showed that endothelial cell activation leads to neutrophil transmigration, supported by the sequential roles of ICAM-2, JAM-A and PECAM-1[29]. We are not aware of previous investigations that link ICAM-2 to prognosis of stable CHF patients. We show that rate of change of ICAM-2 independently predicts adverse clinical outcome. This suggests that prognosis differs between patients with stable ICAM-2 values and patients with increasing ICAM-2 values. CDH5 is an endothelial transmembrane glycoprotein and is the major molecule for cell–cell adhesion that forms adherens junctions[9]. Shedding of CDH5 into the circulation is associated with severe acute kidney injury and with more severe organ dysfunction in patients with sepsis[30] and increased levels of soluble CDH5 were associated with poor outcome in severe sepsis[31]. In cardiovascular research, elevated levels of CDH5 have also been reported to be associated with coronary atherosclerosis[32]. Based on our results, CDH5 may be of use as a biomarker that reflects on-going inflammation and indicates impending adverse events in CHF patients. JAM-A is involved in the regulation of vascular permeability[27] and genetic deletion and blockade of JAM-A generally results in increased permeability of endothelial cells[33]. JAM-A is also thought to be required for movement of leukocytes toward sites of inflammation[34] and it may be considered as a marker of acute endothelial activation and dysfunction[35]. This is in line with our findings; we demonstrate that repeatedly measured levels of JAM-A show a numerically strong independent association with the PE. The significant role of PECAM-1 in platelet aggregation and migration of leukocytes through the endothelium[36] is interesting in the context of CHF. PECAM-1 has been suggested as a sensitive marker providing early diagnostic aid in acute coronary syndromes[37]. In heart failure research, soluble PECAM-1 was found to be

elevated in the majority of patients with severe CHF[38]. However, we did not find an association of PECAM-1 with prognosis in our CHF cohort.

SELP is of great interest because of its key role in interactions between platelets, leucocytes, and endothelium[39]. Abnormal surface SELP expression[40,41] and soluble SELP levels[42] have been reported in decompensated heart failure, suggesting persistent platelet activation. Regarding their prognostic value, however, levels of soluble SELP, platelet surface SELP, and total platelet SELP did not determine prognosis[43] and our results support these findings. Ep-CAM, CNTN1, ITGB2, and SELE also showed negative results in our study.

Less is known about the other biomarkers in relation to CHF. For example, C1qR is a transmembrane receptor once thought to be only a receptor for C1q, but is now thought to play a role in endothelial cell adhesion[44]. The up-regulation of this receptor by inflammatory mediators and the ability of complement component C1q itself to increase ICAM-1 expression suggest a potential role for the receptor in vascular inflammation and immune injury[45]. To the best of our knowledge, C1qR has never been linked directly to prognostication in CHF patients. In our study, repeatedly measured levels of this marker were independently associated with the PE. EPHB4 serves as receptor for its transmembrane ligand ephrin-B2. Both are specifically expressed on arterial and venous endothelial cells. Hamada et al. concluded ephrin-B2 forward signalling and EPHB4 reverse signalling differentially affect cell adhesion and migration between arterial and venous endothelial cells[46]. We found that both level and slope analysis of EPHB4 were significantly associated with the endpoint, even after adjusting for clinical factors.

While the 263 patients included in our investigation were ambulatory and stable, it has been advocated that grouping of HF patients should not be approached only based on symptoms[47], nor on ejection fraction solely[48]. Definitions have been described to identify more advanced disease HF (AdHF), i.e., patients with worsening clinical condition, high rates of re-hospitalization and mortality (meaning a condition where standard treatments are inadequate and additional interventions must be applied; these patients are suitable for LVAD), as well as end-stage heart failure (patients for which advanced therapies, such as LVAD, is contraindicated and palliative cares should be pursued)[49]. In post-hoc analyses, based on our available data, we identified at least 57 patients who might be

categorized into these two groups at baseline; given their ambulant condition most likely the AdHF group. Thirty of them eventually experienced an endpoint during follow-up. Compared to the other 206 patients, these 57 patients were older, had a higher heart rate, lower systolic and diastolic blood pressure, had higher NT-proBNP, hsTnT and eGFR levels, and were more likely to have prior CVA/TIA and diabetes mellitus. Malfunction of other organs could affect prognosis[51], and, therefore, differences in such risk factors should be taken into account (as for example also highlighted in a recent study about the role of oxidative stress and vascular inflammation in diabetic patients which could result in myocardial infarction[50]). Since we adjusted our current analyses of the association between circulating biomarkers of cell adhesion and clinical outcomes for variables such as diabetes mellitus and atrial fibrillation, we believe we have accounted for this type of confounding as much as we could in this observational study.

Our study has some limitations. First, because of efficiency reasons, we did not use all 1984 available trimonthly samples, but selected 3 samples for patients with a PE (baseline and last 2 prior to the PE), and 2 samples for event-free patients, resulting in 567 samples. Our previous investigations using all samples demonstrated that most of the examined biomarkers show an increase shortly prior to the incident adverse event. Thus, we believe that with our approach we retain the most informative measurements while enhancing efficiency. Second, as described before[15,52], our cohort comprised mainly HF patients with a reduced ejection fraction. This can most likely be attributed to the fact that in the Netherlands, most HF patients with a preserved ejection fraction are treated in secondary referral centers or by the general practitioner. Finally, we used biomarker values in Normalized Protein Expression (NPX) Units, i.e., relative units. While these values can be used for comparing patients and changes over time within a patient, for clinical applications absolute concentrations are recommended.

In conclusion, the present study demonstrates that serial measurements of C1qR, CDH5, CHI3L1, EPHB4, ICAM-2 and JAM-A are independently associated with clinical adverse events in patients with CHF, suggesting that markers of cell adhesion could be useful for individual risk profiling. These biomarkers are also interesting for future therapeutic purposes, as CAMs may be used as targets to inhibit vascular inflammation and endothelial dysfunction. Further studies are warranted to confirm these associations, to investigate whether a combination of

different markers (for example C1qR, CHI3L1 and JAM-A) may improve prognostication and to better elucidate the pathophysiological role of cell adhesion in CHF.

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Chapter 13

Summary and general discussion

Summary and general discussion

The aims and goals of this thesis are described extensively in **Chapter 1**. Briefly, we investigated the incremental predictive value of repeating biomarker measurements during follow-up compared to a single 'baseline' measurement in patients with acquired heart disease. In addition, we investigated the variability of several key blood biomarkers when measured repeatedly post-acute coronary syndrome (ACS) patients that clinically stabilized.

Chapter 1 also describes the two observational cohort studies which formed the basis of the manuscripts that compose this thesis. The BIOMArCS study included 844 patients with ACS. They underwent a median of 17 (25^{th} to 75th percentile 12 to 20) repeated blood samples in the first year after the index ACS. The study endpoint comprised recurrent ACS or cardiac death and was reached by 45 patients. Bio-ShiFT included 263 patients with chronic heart failure (HF) who underwent a median of 9 (25^{th} to 75^{th} percentile 5 to 10) blood samples during a median of 2.2 years follow-up. The study endpoint comprised a combination of hospitalization for HF, heart transplantation, placement of a left ventricular assist device, or cardiac death and was reached by 70 patients.

In **part I** of this thesis, based on the BIOMArCS data, we investigated the post-ACS kinetics and variability of several key cardiac blood biomarkers when measured repeatedly in stable patient who had an ACS.

Cardiac high-sensitivity troponins (hsTn) I and T are mostly known for their use as part of the diagnosis of myocardial infarction. For this purpose, the performance of hsTnI and hsTnT are both very good, and the results of the two biomarkers are practically interchangeable. However, whether the two biomarkers act the same outside the context of an acute myocardial infarction is less well investigated, particularly in post-ACS patients. In **Chapter 2**, described and compared the post-ACS kinetics of hsTnI and hsTnT, using hsTnI and hsTnT measurements from 191 BIOMArCS patients that remained free from adverse cardiac events during one year of follow-up. In addition, we compared the average hsTnI and average hsTnT concentration, and the intra-individual and inter-individual variation for both biomarkers in the 6-12 months post-ACS timeframe when patients were considered to have stable coronary artery disease (CAD).

Compared to hsTnT, HsTnI serum concentrations peaked higher after the index ACS (median 3506ng/L vs. 494ng/L, p<0.001) and were quicker below the biomarker-specific upper reference limit (URL) of normal (mean 16 vs. 19 days, p<0.001). In the post 6 months samples, hsTnI and hsTnT showed only modest correlation (rho-spearman 0.60), whereas the average hsTnT concentration was 5 times more likely to be above the URL than hsTnI. The intra-individual variation of hsTnI and hsTnT were 14.0% and 18.1%, while inter-individual variation were 94.1% and 75.9%, respectively. Given the large variation in hsTnI and hsTnT serum concentrations between patients, with little variation within the patient, it makes sense to use individualized patient-specific reference values instead of one reference value for the entire population. We showed that such a patient-specific reference value can be derived for both hsTnI and hsTnT in the vast majority of the patients using only the first two post 30-day measurements. Such patient-specific reference value could potentially fine-tune future diagnostic processes, especially if the patients exhibit chronically elevated hsTn serum concentrations.

In **Chapter 3**, we investigated the post-ACS kinetics and variability of high-sensitivity C-reactive protein (hs-CRP), N-terminal pro-brain natriuretic peptide (NTproBNP) and soluble ST2 (sST2). All three markers have been proposed as prognostic markers in ACS. However, their parameters of variability in stable ACS patients had not yet been investigated. Nevertheless, this is an important character of a biomarker, as biomarker blood concentrations are not only influenced by the patient's medical condition, but also by the analytical imprecision of the test and by biological variation. In individualized risk prediction, if only a single measurement is used, large variability from the patient's habitual value might lead to incorrect risk classification and thus underestimation or overestimation of the risk of secondary events.

We found considerable variability of hs-CRP and NT-proBNP in our asymptomatic and clinically stable post-ACS patients. In contrast, repeatedly measured sST2 concentration showed little within-patient variability. We concluded that for personalized risk models in stable post-ACS patients using a single measurement, out of the three investigated markers, sST2 might be the most useful biomarker, given the low within-subject variation.

Both the European Society of Cardiology (ESC) and the American Heart Association (AHA) have recently put out new guidelines regarding lipid-lowering with stringent treatment goals, particularly in high-risk patient such as post-ACS patients. The ESC/EAS 2019 guideline recommends low-density lipoprotein cholesterol (LDL-C) reduction of 50% from baseline level (if statin naive), and an LDL-C <1.4 mmol/L. The AHA/ACC 2018 guideline advocates to target for LDL-C <1.8 mmol/L. Patients with LDL-C above these treatment goal should get intensified treatment. In **Chapter 4**, we described to what extent the within-patient variability of LDL-C could influence the effects of lipid-lowering treatment. We measured the LDL-C concentrations in all samples taken after 30 days of the index ACS of 157 BIOMArCS patients that remained endpoint-free and had no changes in statin treatment during follow-up. We found that changes up to 30% in consecutive LDL-C measurements from the same patient could be explained by analytical and biological variation. Obviously, these variations may then inappropriately (re)classify patients above or below the treatment threshold, and, thus, will lead to over- or under- treatment. We feel that this problem warrants much more critical appraisal in clinical practice than currently given.

In **Part II**, we used the data available from the BIOMArCS study for investigating the prognostic value of several blood biomarkers, both established and new emerging ones, in patients with ACS. Particularly, we investigated if repeated measurements of these biomarkers could help predict recurrent ACS or cardiac death.

In **Chapter 5**, we describe the relationship between repeatedly measured sST2 and recurrent ACS or cardiac death. sST2 is an interleukin receptor that is upregulated in response of cardiac stress and promotes adverse remodeling of cardiomyocytes. It is a biomarker mostly used in HF but has also shown potential as a predictor in ACS populations. However in most of the studies investigating the prognostic potential of ST2 in ACS patients, the endpoint of interest comprised a combination of heart failure and cardiac death. Less is known about the relationship between ST2 and thrombo-embolic events in this specific population.

We found that sST2 was slightly, but consistently, higher in patients who reached the endpoint during the year of follow-up than in patients who remained endpoint free (29.6 ng/ml versus 33.3 ng/ml, p-value 0.052). After adjusted for the GRACE-score, higher sST2 concentrations remained significantly associated with recurrent

ACS or cardiac death (adjusted hazard ratio (aHR) [95% confidence interval (CI)] per standard deviation (SD) increase: 1.64 [1.09 to 2.34]). We could not identify a rise of sST2 in anticipation of the study endpoint. Also, the prognostic performance of the repeated measurements showed very little added value when compared to a single measurement randomly selected from the available samples. These results suggest that sST2 might not be the best biomarker for prognostication of thrombo-embolic events in (post)ACS patients.

In **Chapter 6**, we explored the association between temporal patterns of myeloperoxidase (MPO) and galectin-3 (GAL-3) in relation to recurrent ACS or cardiac death . MPO and GAL-3 are pro-inflammatory proteins that promote plaque vulnerability through various mechanisms such as nitric oxide catalyzation, foam cell formation and vascular smooth muscle cell dedifferentiation. Although MPO and (to a somewhat lesser extent) GAL-3 were elevated early after an ACS, they did not show steady or sudden elevations prior to a new ACS. In addition, post-ACS patients who experienced a recurrent event within one year were not characterized by elevated levels of these pro-inflammatory biomarkers compared to patients that remained event-free. We thus concluded that MPO and GAL-3 appeared unsuited for prognosis monitoring after ACS.

Impaired renal function is known to predict mortality in ACS. The post-ACS kinetics however had not yet been described though. In addition, it was unknown if repeated measurements of biomarkers reflecting impaired renal function would carry prognostic value. Hence, in **Chapter 7**, we explored the post-ACS kinetics and prognostic values of using serial measurements of creatinine and cystatin C (cysC). By design, the BIOMArCS study excluded patients with estimated glomerular filtration rate (eGFR) Cr<30 ml/min/1.73 m2. The main findings from our study were that CysC concentrations indicated deterioration of renal function earlier than creatinine did (CysC peaked on day 3, versus day 6 for creatinine), and that higher CysC concentrations, but not creatinine concentrations, predicted recurrent ACS or cardiac death independently of the GRACE score within the first year after index ACS (aHR [95% CI] per SD ncrease: 1.68 [1.03 to 2.74]). Both CysC and creatinine concentrations stabilized to levels below the URL within two weeks after the index ACS.

Many novel biomarkers that may theoretically influence the atherosclerotic process have been investigated as predictors for recurrent ACS in recent years. In **Chapter 8,** we explored the correlation between novel lipidomic biomarkers and their association with ACS in the BIOMArCS population. Using nuclear magnetic resonance, we determined the levels of 151 lipid metabolites in a median of 7 blood samples per patient. After correction for multiple testing, high concentrations of extremely large very low density lipoprotein particles (aHR [95%CI] per SD increase: 1.60 [1.25 to 2.08]), very large VLDL particles (aHR [95%CI] per SD increase: 1.60 [1.25 to 2.08]) and large VLDL particles (aHR [95%CI] per SD increase 1.56 [1.22 to 2.05]) were significantly associated with recurrent ACS or cardiac death.

In **Part III**, we investigated the prognostic potential of repeated biomarker measurements in HF patients, including echocardiograms and blood biomarkers. For these studies we used the data available from the Bio-SHiFT study.

Both ACC/AHA guidelines and ESC guidelines indicate that echocardiography is the single most useful test in the diagnosis of HF. It can be used to detect structural abnormality, systolic dysfunction, diastolic dysfunction, or a combination of these abnormalities either in stable phase or during symptoms of HF. In addition, echocardiography is relatively inexpensive, has a high feasibility, and is capable of producing robust and simple measurements. Current HF guidelines do not recommend periodically repeating echoes in otherwise stable HF patients. Reassessing myocardial structures and functions is only warranted when patients present with symptoms of worsening HF or experience any important cardio-vascular event, prior to device implantations and during exposure to cardiotoxic therapies. We, however, hypothesized that repeated echocardiograms could provide incremental prognostic value over a single measurement and might be useful for identifying periods of high-risk for hospitalization or other major cardiac adverse events. In **Chapter 9**, we followed 106 stable HF patients from the Bio-SHiFT study for a median of 2.3 years in which 332 echocardiograms were performed with a median [25th to 75th percentile] of 3 [2 to 4] per patient. A total of 25 patients reached the study endpoint.

Both the single measurements and the longitudinal trajectories of the measured echo variables were significantly associated with the endpoint (aHR Cox model

[95% CI] vs aHR joint model [95% CI]): Left ventricular (LV) ejection fraction, 1.47 [0.93 to 2.31] vs 1.77 [1.13 to 2.93]; diastolic LV diameter, 1.64 [1.09 to 2.47] vs 1.68 [1.12 to 2.57]; systolic LV diameter, 1.72 [1.10 to 2.69] vs 1.68 [1.13 to 2.63]; systolic left atrial diameter, 1.88 [1.18 to 3.00] vs 2.60 [1.48 to 4.97]; E/A ratio, 2.73 [1.42 to 5.26] vs 3.87 [1.75 to 10.13]; and E/e' ratio, 2.30 [1.38 to 3.84] vs 2.99 [1.68 to 6.19]. However, the longitudinal trajectories of the investigated parameters, constructed using the repeated echocardiograms, failed to reveal deterioration prior to events and the aHR in the single measurement models and the repeated measurement models were comparable. We thus concluded that, regular echocardiographic monitoring of systolic or diastolic LV diameter and function within this time frame does not carry incremental prognostic information over a single baseline measurement.

In **Chapter 10**, we examined the association between serial simultaneous measurements of echocardiographic parameters and hsCRP, NT-proBNP, and hs-TnT in 117 HF patients with ejection fraction ≤50%. Median follow-up was 2.2 years (25^{th} to 75th percentile 1.5 to 2.6). We performed up to six follow-up evaluations with 55% of patients having at least three evaluations performed. A model containing all three biomarkers revealed that doubling of NT-proBNP was associated with a decrease in left ventricular ejection fraction by 1.83 (95%CI: −2.63 to −1.03)%; relative increase in mitral E/e' ratio by 12 (95% CI: 6 to 18)%; relative increase in mitral E/A ratio by 16 (95% CI:9 to 23)%; decrease in tricuspid annular plane systolic excursion by 0.66 (95% CI: −1.27 to −0.05) mm; rise in tricuspid regurgitation peak systolic gradient by 2.74 (95% CI: 1.43 to 4.05) mmHg; and increase in left ventricular and atrial dimensions. Hs-TnT and hs-CRP showed significant associations with some echocardiographic, but after adjustment for the other biomarkers the associations were not significant. Our results support further studies on NT-proBNP as a surrogate marker for hemodynamic congestion and herewith support its potential value for therapy guidance.

In **Chapter 11** and **Chapter 12**, we associated 4 longitudinally measured biomarkers of fibrinolysis and 12 longitudinally measured biomarkers of adhesion molecules with cardiovascular (CV) events in patients with chronic HF. The biomarkers were measured using an Olink Proteomics multiplex assay in a total of 567 samples belonging to 263 ambulant stable HF patients included in the first wave of the Bio-SHiFT study.

Thrombotic factors are known to be upregulated in HF and more severe upregulation is associated with the poorer outcome of the disease. Whether the same is true for fibrinolytic factors, is less well investigated. Thus, in **Chapter 11**, we investigated the prognostic value of 4 factors involved in the process of fibrinolysis in HF patients, namely: Plasminogen activator inhibitor-1 (PAI), Tissue-type plasminogen activator (tPA), Urokinase-type plasminogen activator (uPA), and soluble urokinase plasminogen activator surface receptor (suPAR). We found that longitudinally measured PAI-1, uPA, and suPAR were independently associated with adverse cardiac events after correction for clinical characteristics (aHR [95%CI]) per SD increase of 2.09 [1.28 to 3.45]) for PAI-1, 1.91 [1.18 to 3.24] for uPA, and 3.96 [2.48 to 6.63] for suPAR. Serial measurements of tPA were not significantly associated with the event after correction for multiple testing. We concluded that PAI-1, uPA and suPAR might be important markers for improving and personalizing feature HF surveillance and treatment monitoring.

In **Chapter 12**, we investigated plasma levels of 12 adhesion molecules and their association with cardiac endpoints in chronic HF. Cardiovascular inflammation and vascular endothelial dysfunction are involved in chronic HF, and cellular adhesion molecules are considered to play a key role in these mechanisms. Repeatedly measured levels of Complement component C1q receptor (C1qR), Cadherin 5 (CDH5), Chitinase-3-like protein 1 (CHI3L1), Ephrin type-B receptor 4 (EPHB4), Intercellular adhesion molecule-2 (ICAM-2) and Junctional adhesion molecule A (JAM-A) were independently associated with the PE. Their rates of change also predicted clinical outcome. Level of CHI3L1 was numerically the strongest predictor with aHR [95%CI] of 2.27 [1.66 to 3.16] per SD difference in level, followed by JAM-A (2.10 [1.42 to 3.23]) and C1qR (1.90 [1.36 to 2.72]). If future research with direct measurements confirms our findings, these molecules could be useful for identifying high-risk patients and high-risk periods.

Conclusions and future perspectives

Previous research using (blood) biomarkers to predict adverse outcomes in patients with acquired heart disease has mostly used one-time measurement and has produced useful prediction models[1,2]. In addition, it has provided new insights into both the treatment and pathophysiology of ACS and HF. However, a one-time measurement approach also has obvious limitations. As time since the

measurement increases, the relationship between the biomarker level measured and the health status at that time is likely to diminish. Moreover, even if a prediction model has good discriminative value during a longer follow-up, it cannot be used to predict when during follow-up patients are most vulnerable and the event is most likely to occur. Finally, as shown in part I, fluctuations of measured biomarker concentrations caused by both analytical and biological variation can lead to misclassification in patients' risk profiles if the biomarker is only measured once.

Using repeated biomarker measurements has theoretical advantages when compared to a one-time measurement. With repeated measurements, we can potentially create models that can predict more accurately which patient is vulnerable for a new event as well as predict when in time this patient is most vulnerable for adverse events. If such a model has a proven good performance and is well validated, it could impact the way outclinic patients are treated. In an ideal situation, patients would have their blood taken regularly, and the biomarkers of interest are measured. These biomarker concentrations are then entered into the repeated measurement prediction model and a new updated risk for adverse events for the upcoming period for the patient is produced by the model[3]. This risk can then be used by the cardiologist to pre-emptively adjust the medication and prevent hospitalization or other adverse events (see Figure 13.1).

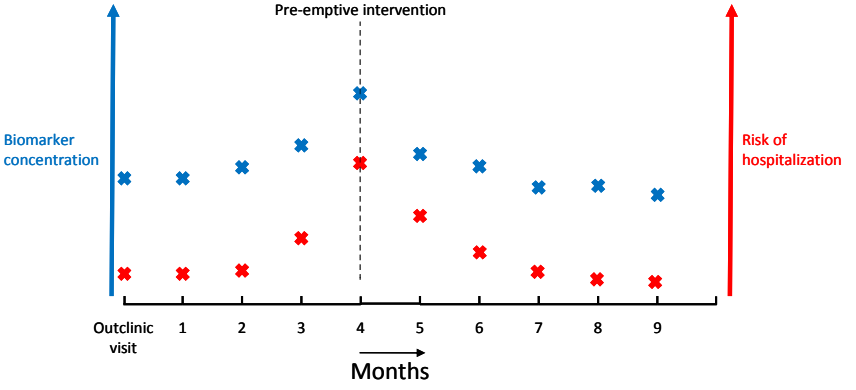

Figure 13.1 Example of potential use of repeated blood sampling during follow-up of an outclinic patient. Blue crosses depict biomarker concentrations; red crosses depict the corresponding risk of hospitalization. The dotted line represents a pre-emptive intervention from the treating clinician.

In HF patients, research with repeated measurements has indeed shown promising results for risk prediction. HF can be characterized as a process of long-term deterioration involving numerous pathological pathways. It is known that biomarkers reflecting these pathways are clearly associated with the current clinical status of the patient. The most well-known example is NT-proBNP, which is measured in clinical practice for estimating severity of decompensation or current HF status. In the current thesis, but also in previous work from our research group, we have shown that repeatedly measuring biomarkers concentration during follow-up of HF patients gives us incremental prognostic information when compared to a single measurement. For several biomarkers including well-known markers NT-proBNP, ST2, and Cystatin C, it has been shown that the longitudinal pattern during follow-up can be used to predict both high-risk patients as well as high risk periods[4-6]. A future step in this line of research could be to investigate whether using such a HF prediction model in clinical practice could lead to early detection of high-risk periods, which in turn allows for appropriate medication changes and ultimately the prevention of hospital admissions. Although much more invasive and expensive than a repeated biomarker approach, the CardioMEMS HF system has in some respects already shown the feasibility and potential of such an approach. CardioMEMS is a device which continuously measures the pulmonary artery pressure (PAP). A lower PAP is associated with improved clinical outcomes. Using remote monitoring the cardiologist can judge the current status of heart failure and when PAP rises, alter treatment well before the patient experiences symptoms. In clinical trials, the CardioMEMS HF system has shown to reduce HF hospitalizations and improve the quality of life of the patients[7,8].

In patients with ACS, we have seen less promising results using repeated measurements for the prediction of adverse CV events. ACS is a sudden manifestation of the more chronic process of CAD. The pathophysiology of an ACS is well described and involves rupturing of an existing plaque causing blood to cloth and finally occlusion of a coronary artery. Despite the high-frequency sampling, BIOMArCS was unable to predict such events, as none of the investigated biomarkers evidently increased prior to re-ACS. Several biomarkers did show discriminative power between high-risk and low-risk patients for recurrent events during the year of follow-up[9-11] however the incremental value of the high frequency sampling compared to a single measurement seems limited and does not outweigh the extra efforts.

Despite that the BIOMArCS study was not able to foresee the timing of reACS, it did (again) show that biomarkers reflecting the severity of the underlying CAD can be used to identify high-risk patients during the follow-up of 1 year. Future studies should therefore look into whether a low frequency sampling (e.g. once every 6 months) of biomarkers reflecting the state of CAD could contribute to a continuous risk assessment of post-ACS patients. If it does, these results could be used to personalize treatment for patients with chronic CAD

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Epiloque

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Dankwoord

Nederlandse samenvatting

Voor dit proefschrift hadden we twee verschillende hoofdvragen. Ten eerste wilde we onderzoeken of het herhalen van biomarkermetingen tijdens de follow-up van een patient met verworven hartziekten, leidt tot nauwkeurigere voorspelmodellen voor toekomstige hartklachten dan traditionele voorspelmodellen waarbij één enkele 'baseline' meting wordt gebruikt. Ten tweede wilden we kijken hoe stabiel de gemeten concentraties van enkele veel gebruikte bloedbiomarkers zijn gedurende de follow-up in klinisch stabiele patienten. Voor onze onderzoeken hebben we ons geconcentreerd op patienten die een acuut coronair infarct (ACS) hebben gehad en op patiënten met hartfalen (HF).

Voor de verschillende studies die we hebben uitgevoerd hebben we twee observationele cohortstudies gebruikt. De eerste studie is de BIOMArCS-studie. In deze studie werden 844 patiënten met ACS geincludeerd. Gedurende het eerste jaar na hun ACS werd er mediaan 17 (25e tot 75e percentiel 12 tot 20) keer bloed van hen afgenomen. Het eindpunt van de studie omvatte de combinatie van een recidief ACS of dood door een cardiale oorzaak en werd bereikt door 45 patienten. De tweede studie is de Bio-ShiFT-studie. Hierin werden 263 patiënten met chronisch HF geincludeerd waarbij een mediaan van 9 (25e tot 75e percentiel 5 tot 10) keer bloed werd afgenomen gedurende een mediane follow-up van 2.2 jaar. Het studie-eindpunt was een combinatie van ziekenhuisopname voor HF, harttransplantatie, plaatsing van een left ventricular assist device of dood door cardiale oorzaak en werd bereikt door 70 patiënten.

In **deel I** van dit proefschrift hebben we, op basis van data uit de BIOMArCS, het verloop in concentraties direct na een ACS van enkele belangrijke bloedbiomarker beschreven. Daarnaast hebben we gekeken naar de variabiliteit in herhaalde metingen van deze biomarkers als deze over de tijd herhaald worden gemeten in verder klinisch stabiele patienten.

Cardiale high-sensitivity troponine (hsTn) I en T worden klinisch vooral gebruikt voor de diagnostiek bij een hartinfarct. Voor dit doel zijn de prestaties van hsTnI en hsTnT beide erg goed en zijn de resultaten van de twee biomarkers praktisch uitwisselbaar. Het is echter minder goed bekend of de twee biomarkers ook buiten de context van een ACS uitwisselbaar zijn. Zeker bij patienten die eerder een ACS

hebben gehad, is dit nooit goed onderzocht. In **Hoofdstuk 2** wordt het verloop in concentraties van hsTnI en hsTnT beschreven en vergeleken direct na een ACS. Hiervoor gebruikten we de data van 191 BIOMArCS-patiënten die gedurende één jaar follow-up klinisch stabiel bleven. We hebben tevens de gemiddelde hsTnI- en gemiddelde hsTnT-concentratie gedurende de follow-up vergeleken, en de intra-individuele en interindividuele variatie voor beide biomarkers bepaald in de periode tussen 6 en 12 maanden na het ACS. We hebben deze specifieke periode gekozen omdat we er vanuit gaan dat de patienten op dat moment zowel klinisch als biochemisch stabiel zouden zijn en we eigenlijk kunnen spreken van stabiele coronaire hartziekte (CAD).

Vergeleken met hsTnT, piekt hsTnI hoger na de index ACS (mediane concentratie 3506 ng/l versus 494 ng/l, $p<0.001$) en was hsTnI sneller onder de normale referentiewaarde (URL) (gemiddeld 16 versus 19 dagen, $p<0.001$). In het bloed afgenomen in de periode tussen 6 en 12 maanden na het ACS was er slechts een matige correlatie tussen de gemeten hsTnI en hsTnT concentraties te vinden (rho-Spearman 0.60). Bij het vergelijken van de gemiddelde concentratie hsTnT binnen iedere patient met de gemiddelde concentratie van hsTnI binnen dezelfde patient, zagen we dat hsTnT 5 keer vaker boven de URL lag dan bij hsTnI. De variatie binnen metingen van dezelfde patient voor hsTnI en hsTnT was 14.0% en 18.1%. De variatie tussen verschillende patienten was echter veel groter, namelijk 94.1% voor hsTnI en 75.9% voor hsTnT. Gezien er weinig variatie is van hsTn-serumconcentraties in klinische stabiele patienten maar er wel veel grote verschillen zijn in gemiddelde concentraties tussen verschillende patienten, is het eigenlijk niet logisch om één referentiewaarde te hebben voor de hele populatie. Het lijkt juist zinvoller om geïndividualiseerde patiëntspecifieke referentiewaarden te gebruiken. Met onze data, hebben we aangetoond dat een dergelijke patiëntspecifieke referentiewaarde voor zowel hsTnI als hsTnT bij het overgrote deel van de patiënten kan worden bepaald met behulp van de eerste twee metingen genomen na de eerste maand. Met een patiëntspecifieke referentiewaarde zou mogelijk het diagnostische proces rondom nieuwe ACS kunnen verfijnen, vooral in de patiënten die in de stabiele fase al chronisch verhoogde hsTn-serumconcentraties hebben.

In **Hoofdstuk 3** onderzochten we het verloop na ACS en de variabiliteit van high-sensitive C-reactive protein (hs-CRP), N-terminal pro-brain natriuretic peptide (NT-

proBNP) en soluble ST2 (sST2). Alle drie de markers worden in de literatuur genoemd als potentiele kandidaten om de prognose in patienten met een ACS te bepalen maar de variabiliteit van deze biomarkers in deze specifieke (post-ACS) populatie was nog niet onderzocht. Wij achten dit echter wel een belangrijk kenmerk van een biomarker, aangezien de bloedconcentraties van biomarkers niet alleen worden beïnvloed door de medische toestand van een patiënt, maar ook door de onnauwkeurigheid van de test en door biologische variatie. Als bij een geïndividualiseerde risicovoorspelling slechts één enkele meting wordt gebruikt, kan een grote variabiliteit rondom de gemiddelde waarde van de patiënt leiden tot een onjuiste risicoclassificatie en dus tot een onderschatting of overschatting van het risico op nieuwe klachten.

De belangrijkste bevindingen van ons onderzoek waren dat er een aanzienlijke variabiliteit werd gezien in herhaald gemeten serumconcentraties van hs-CRP en NT-proBNP in verder asymptomatische en klinisch stabiele post-ACS-patiënten. Daarentegen vertoonde herhaaldelijk gemeten sST2-concentratie weinig variabiliteit binnen de patiënt. We concludeerden dat voor een conventioneel risicomodel voor stabiele post-ACS patiënten, dat gebruikt maakt van een enkele 'baseline' meting, sST2 de meest bruikbare marker zou zijn van de drie onderzochte markers.

Zowel de European Society of Cardiology (ESC) als de American Heart Association (AHA) hebben recent nieuwe richtlijnen gepubliceerd waarin strikte behandeldoelen worden gesteld voor patiënten met een verhoogd risico op cardiovasculaire klachten, zoals de BIOMArCS patiënten. De ESC/EAS 2019-richtlijn beveelt een low-density lipoprotein-cholesterol (LDL-C) -reductie aan van tenminste 50% van het uitgangsniveau (als de patient nog geen statine gebruikte), en daarnaast LDL-C concentratie van minder dan 1,4 mmol/L. De AHA/ACC 2018-richtlijn pleit ervoor om te streven naar een LDL-C concentratie van 1,8 mmol/L in de hoog-risicogroep. Patiënten met LDL-C concentratie boven deze behandeldoelen dienen een intensievere behandeling te krijgen in de vorm van meer medicatie. In **Hoofdstuk 4** hebben we beschreven in hoeverre de variabiliteit van LDL-C concentraties binnen een patiënt effect zou kunnen hebben op de behandeling die hij krijg om zijn lipidenprofiel te verbeteren. We hebben hiervoor de LDL-C-concentraties gemeten van alle beschikbare bloedmonsters, afgenomen vanaf tenminste één maand na het ACS, van 157 klinisch stabiele BIOMArCS-

patiënten die gedurende de follow-up geen veranderingen hadden in hun statinebehandeling. We ontdekten dat veranderingen tot 30% in opeenvolgende LDL-C-metingen binnen dezelfde patiënt verklaard konden worden door alleen de analytische en biologische variatie. Logischerwijs kan een verschil van 30% boven of onder de gemiddelde LDL-C concentratie van een patient er voor zorgen dat zij boven of onder de strenge behandeldoelen van de nieuwe richtlijn vallen. Dit zal dan weer leiden tot over - of onderbehandeling. Op basis van deze data zijn wij van mening dat dit variabiliteit van LDL-C in de klinische praktijk problemen zal geven en dat dit probleem onderbelicht is in de huidige richtlijn.

In **deel II** van dit proefschrift, hebben we met behulp van de data van de BIOMArCS-studie de prognostische waarde van verschillende bloedbiomarkers onderzocht bij patiënten met ACS. Hierbij hebben we ons in het bijzonder gefocust op de vraag of herhaalde metingen van deze biomarkers meerwaarde bieden bij het voorspellen van een recidief-ACS of cardiale dood.

In **Hoofdstuk 5** beschrijven we de relatie tussen herhaaldelijk gemeten sST2 en het risico op een recidief-ACS of cardiale dood. sST2 is een biomarker die meestal wordt gebruikt in HF maar heeft ook wordt genoemd als potentiele kandidaat om voorspellingen te doen bij patiënten met ACS. In de meeste onderzoeken die het prognostische potentieel van ST2 bij ACS-patiënten onderzochten, bestond het eindpunt echter uit een combinatie van hartfalen en hartdood. Er is minder bekend over de relatie tussen ST2 en trombo-embolische events zoals het recidief ACS bij deze specifieke populatie.
We ontdekten dat sST2-concentraties gemiddeld gezien licht verhoogd waren bij patienten die het eindpunt bereikten tijdens het jaar van follow-up ten opzichte van patiënten die eindpuntvrij bleven (29,6 ng/ml versus 33,3 ng/ml, p-waarde 0,052). In een model gecorrigeerd voor de GRACE-score bleken hogere sST2-concentraties significant geassocieerd met een recidief-ACS of dood door cardiale oorzaak (gecorrigeerde hazard ratio (aHR) [95% betrouwbaarheidsinterval (BI)] per standaarddeviatie (SD) toename: 1.64 [1.09 tot 2.34]). We zagen helaas geen stijging van het sST2 voorafgaand aan het studie-eindpunt. Daarnaast was de prognostische waarde van de herhaalde meting maar marginaal beter dan de prognostische waarde van een enkele sST2 meting. Onze resultaten suggereren dat het herhaald meten van sST2 niet de beste keuze is voor het prognosticeren van trombo-embolische voorvallen bij (post) ACS-patiënten.

In **Hoofdstuk 6** hebben we de associatie onderzocht tussen herhaaldelijk gemeten myeloperoxidase (MPO) en galectin-3 (GAL-3) aan de ene kant en het risico op recidief-ACS en dood door cardiale oorzaak aan de andere kant. MPO en GAL-3 zijn pro-inflammatoire eiwitten die de kwetsbaarheid van coronaire plaques bevorderen door middel van verschillende mechanismen. Hoewel MPO en (in iets mindere mate) GAL-3 vroeg na een ACS verhoogd waren, vertoonden ze geen stabiele of plotselinge stijging voorafgaand aan een recidief-ACS tijdens het jaar follow-up. Bovendien werden de post-ACS-patiënten die binnen een jaar een recidief-ACS doormaakten of stierven door een cardiale oorzaak, niet gekenmerkt door verhoogde niveaus van MPO of GAL3. We concludeerden dus dat deze pro-inflammatoire biomarkers ongeschikt zijn voor het bepalen van de prognose in patiënten die een ACS hebben gehad.

Het is bekend dat een verminderde nierfunctie een belangrijke voorspeller is van mortaliteit bij patiënten met een ACS. Het verloop van de nierfunctie direct na een ACS was echter nog niet beschreven. Bovendien was het nooit onderzocht of herhaald meten van biomarkers die een verminderde nierfunctie weerspiegelen, zou kunnen leiden tot een nauwkeuriger prognostische profiel in deze patientenpopulatie. Daarom hebben we in **Hoofdstuk 7** met behulp van herhaalde metingen, het beloop van creatinine en cystatine C (cysC) na een ACS onderzocht en hebben we de prognostische waarde van deze herhaald gemeten biomarkers bepaald. Belangrijk om hierbij te vermelden is dat bij de opzet van het BIOMArCS-onderzoek ervoor is gekozen om patiënten met een geschatte glomerulaire filtratiesnelheid (eGFR) Cr <30 ml / min / 1,73 m2 uit te sluiten van deelname.

De belangrijkste bevindingen van onze studie waren dat zowel CysC-concentraties en creatinine-concentraties stijgen na een ACS maar CysC bereikt eerder zijn piekwaarde (CysC piekte op dag 3, versus dag 6 voor creatinine). Tijdens de verdere follow-up was het hebben van een hoog CysC geassocieerd met een verhoogd risico op een recidief-ACS of dood door een cardiale oorzaak (aHR [95% BI] per SD-stijging: 1.68 [1.03 tot 2.74]). Bij creatinine werd niet zo'n verband gevonden. Zowel CysC- als creatinine-concentraties stabiliseerden zich binnen twee weken na de index ACS tot niveaus onder de URL.

De afgelopen jaren is er veel onderzoek gedaan naar nieuwe biomarkers die in theorie het atherosclerotische proces zouden kunnen beïnvloeden en waarmee

mogelijk het optreden van nieuwe cardiale ziektes kan worden voorspeld. In **Hoofdstuk 8** hebben we de assocatie onderzocht tussen nieuwe 'lipidomic' biomarkers aan de ene kant en het risico op recidief ACS en dood door cardiale oorzaak aan de andere kant in de BIOMArCS-populatie. Met behulp van nucleaire magnetische resonantie werd het niveau van 151 veschillende lipidenmetabolieten bepaald in mediaan 7 bloedmonsters per patiënt, afgenomen gedurende het jaar follow-up. Na correctie voor meervoudige testen, bleek dat hoge concentraties van extreem grote lipoproteïnedeeltjes met zeer lage dichtheid (VLDL) (aHR [95% BI] per SD-toename: 1.60 [1.25 tot 2.08]), zeer grote VLDL-deeltjes (aHR [95% BI] per SD-toename: 1.60 [1.25 tot 2.08]) en grote VLDL-deeltjes (aHR [95% BI] per SD-toename 1.56 [1.22 tot 2.05]) significant geassocieerd was met recidief-ACS en dood door cardiale oorzaak.

Tenslotte hebben we in **deel III** van dit proefschrift, de prognostische waarde van herhaalde bloedbiomarkermetingen en herhaale echocardiogrammetingen in patienten met HF onderzocht. Voor deze onderzoeken hebben we de data uit de Bio-SHiFT-studie gebruikt.
Zowel ACC/AHA-richtlijnen als ESC-richtlijnen schrijven voor dat echocardiografie de meest bruikbare test is om HF te diagnosticeren. Echocardiografie kan worden gebruikt om structurele afwijkingen van het hart, systolische disfunctie, diastolische disfunctie of een combinatie van deze afwijkingen op te sporen. De techniek kan zowel tijdens stabiele ziekte, als tijdens acute symptomen van HF worden gebruikt, het is relatief goedkoop, goed uitvoerbaar en er kunnen robuuste en eenvoudige metingen mee worden uitgevoerd. De huidige HF-richtlijnen bevelen echter het periodiek herhalen van echo's bij verder stabiele HF-patiënten niet aan. Het opnieuw beoordelen van myocardstructuren en -functies zou alleen moeten worden uitgevoerd bij patiënten van wie de symptomen van het HF verslechteren of die andere relevante cardiale klachten hebben, bij patienten die een cardiaal device geimplanteerd krijgen of patienten die cardiooxische chemotherapie. Wij hypothethiseerden echter dat we met herhaalde echocardiogrammen mogelijk beter perioden met een verhoogd risico op ziekenhuisopname of andere ernstige cardiale klachten konden voorspellen. Om deze hypothese te testen, hebben we in **Hoofdstuk 9** 106 stabiele HF-patiënten uit de Bio-SHiFT-studie gevolgd gedurende een mediaan van 2.3 jaar waarin 332 echocardiogrammen werden gemaakt met een mediaan [25e tot 75e percentiel]

van 3 [2 tot 4] per patiënt. In totaal bereikten 25 patiënten het eindpunt van de studie.

Zowel de enkelvoudige nulmetingen als de herhaalde metingen van de gemeten echovariabelen waren significant geassocieerd met het eindpunt (aHR Cox/'baseline'-model [95% BI] vs. aHR herhaalde metingen-model [95% BI]): linkerventrikel (LV) ejectiefractie, 1.47 [0.93 tot 2.31] versus 1.77 [1.13 tot 2.93]; diastolische LV-diameter, 1.64 [1.09 tot 2.47] versus 1.68 [1.12 tot 2.57]; systolische LV-diameter, 1.72 [1.10 tot 2.69] versus 1.68 [1.13 tot 2.63]; systolische linker atriale diameter, 1.88 [1.18 tot 3.00] versus 2.60 [1.48 tot 4.97]; E / A-verhouding, 2.73 [1.42 tot 5.26] versus 3.87 [1.75 tot 10.13]; en E / e'-verhouding, 2.30 [1.38 tot 3.84] versus 2.99 [1.68 tot 6.19]. Met behulp van de herhaalde echocardiogrammen, hebben we het verloop van de onderzochte parameters gedurende de follow-up geschat. Deze lieten echter geen verslechtering zien voorafgaand aan het studie-eindpunt en ook de aHR van de herhaalde meting-modellen waren vergelijkbaar met die van de enkelvoudige nulmeting-modellen. We concludeerden dus daarom dat regelmatige echocardiografische monitoring van de systolische of diastolische LV-diameter en -functie geen grotere prognostische informatie verschaft ten opzichte van een enkele nulmeting binnen dit tijdsbestek.

In **Hoofdstuk 10** onderzochten we de associatie tussen gelijktijdige gemeten echocardiogramparameters en hsCRP, NT-proBNP en hs-TnT. Dit deden we bij 117 HF patienten uit de Bio-SHiFT waarbij gedurende een mediane follow-up was 2.2 jaar (25e tot 75e percentiel 1.5 tot 2.6) tot zes echocardiogrammen en bloedafnames waren uitgevoerd (mediaan van 3). Een model met alle drie de biomarkers onthulde dat een verdubbeling van NT-proBNP geassocieerd was met een afname van de linkerventrikelejectiefractie met 1.83 (95% BI: -2.63 tot -1.03)%; relatieve toename van de mitralis-E / e '-ratio met 12 (95% BI: 6 tot 18)%; relatieve toename van de mitralis-E / A-ratio met 16 (95% BI: 9 tot 23)%; afname in tricuspidalis ringvormig vlak systolische excursie met 0.66 (95% BI: -1.27 tot -0.05) mm; stijging van de systolische piekgradiënt van tricuspidalisinsufficiëntie met 2.74 (95% BI: 1.43 tot 4.05) mmHg; en toename van de afmetingen van de linker hartkamer en het atrium. Hs-TnT en hs-CRP vertoonden significante associaties met sommige echo parameters, maar na correctie voor de andere biomarkers waren de associaties niet langer statistisch significant. Onze resultaten ondersteunen verdere

studies naar NT-proBNP als een surrogaatmarker voor echocardiografische parameters en ondersteunen daarmee de potentiële waarde ervan voor therapiebegeleiding.

In **Hoofdstuk 11** en **Hoofdstuk 12** hebben we het verloop over tijd van 4 herhaald gemeten fibrinolyse factoren en 12 herhaald gemeten biomarkers van adhesiemoleculen geassocieerd met het risico op het HF-eindpunten bij ambulante stabiele HF-patiënten. De biomarkers werden gemeten met behulp van een Olink Proteomics multiplex-assay in 567 bloedmonsters, afgenomen bij 263 patiënten geïncludeerd in de Bio-SHiFT-studie.

Van HF is bekend dat het leidt tot een verhoogd risico op trombose; dit komt door hypercoagulabiliteit van het bloed in combinatie met een trage flow en vaatwandschade. Het risico op trombose is hierbij geassocieerd met de de ernst van het hartfalen. Als verhoogde stollingsneigeing geassocieerd is met ernstiger hartalen, dan is de aan de andere kant fibrinolyse dat misschien ook wel. Om dat uit te zoeken, hebben we in **Hoofdstuk 11** de prognostische waarde onderzocht van 4 factoren die betrokken zijn bij het proces van fibrinolyse in HF-patiënten, namelijk: plasminogen activator inhibitor 1 (PAI), tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and soluble urokinase plasminogen activator surface receptor (suPAR). We ontdekten dat herhaald gemeten PAI-1, uPA en suPAR geassocieerd waren met het Bio-SHiFT studie-eindpunt, zelfs na correctie voor klinische kenmerken (aHR [95% BI]) per SD-toename van 2.09 [1.28 tot 3.45]) voor PAI-1, 1.91 [1.18 tot 3.24] voor uPA en 3.96 [2.48 tot 6.63] voor suPAR. Herhaalde metingen van tPA waren niet significant geassocieerd met de het studie-eindpunt. We concludeerden dat PAI-1, uPA en suPAR belangrijke biomarkers kunnen zijn voor het verbeteren en personaliseren van HF-bewaking en behandelingsmonitoring.

Tenslotte hebben we in **Hoofdstuk 12** plasmaspiegels van 12 adhesiemoleculen bekeken en hun associatie met cardiale eindpunten bij chronische HF. Tijdens chronische HF ontwikkelt zich vaak ook in meer of mindere mate vaatontsteking en vasculaire endotheeldisfunctie en cellulaire adhesiemoleculen worden geacht een sleutelrol te spelen in dit proces. Herhaaldelijk gemeten niveaus van complementcomponent C1q-receptor (C1qR), cadherine 5 (CDH5), chitinase-3-achtig proteïne 1 (CHI3L1), ephrine type-B-receptor 4 (EPHB4), intercellulair

adhesiemolecuul-2 (ICAM-2) en Junctioneel adhesiemolecuul A (JAM-A) waren onafhankelijk geassocieerd met het studie-eindpunt. Het niveau van CHI3L1 was numeriek de sterkste voorspeller met aHR [95% BI] van 2.27 [1.66 tot 3.16] per SD niveauverschil, gevolgd door JAM-A (2.10 [1.42 tot 3.23]) en C1qR (1.90 [1.36 tot 2.72]). Als toekomstig onderzoek met directe metingen onze bevindingen bevestigt, kunnen deze moleculen nuttig zijn voor het identificeren van hoogrisicopatiënten en hoogrisicoperiodes.

Conclusies en aanbevelingen voor verder onderzoek

In eerder onderzoek om nadelige uitkomsten te voorspellen bij patiënten met een verworven hartaandoening werd vooral gebruik gemaakt van éénmalige nulmetingen van (bloed)biomarkers. Dit soort onderzoek heeft geleid tot enkele klinische veelvuldig gebruikte voorspelmodellen binnen de cardiologie. Bovendien heeft dit soort onderzoek met bloedbiomarkers ons nieuwe inzichten opgeleverd in zowel de behandeling als de pathofysiologie van ACS en HF. Een aanpak met een éénmalige nulmeting heeft echter ook duidelijke beperkingen. Naarmate de tijd sinds de meting toeneemt, zal de relatie tussen het toen gemeten biomarkerniveau en de huidige gezondheidsstatus waarschijnlijk afnemen. Zelfs als een voorspellingsmodel met nulmeting wel een goede discriminerende waarde heeft tijdens een langere follow-up, dan nog kan het niet worden gebruikt om te voorspellen wanneer tijdens de follow-up patiënten het kwetsbaarst zijn en de nieuwe cardiale events het meest waarschijnlijk zal plaatsvinden. Tenslotte hebben we aangetoond in **deel I**, dat er bij een éénmalige meting van biomarkerconcentraties op basis van de analytische en biologische variatie, er zeker patienten verkeerd zullen worden gemisclassificeerd.

Het gebruik van herhaalde biomarkermetingen heeft theoretische voordelen in vergelijking met een éénmalige meting in voorspelmodellen. Met herhaalde metingen kunnen we modellen creëren die mogelijk nauwkeuriger kunnen voorspellen welke patiënt kwetsbaar is voor een nieuwe klachten en ook modellen die kunnen voorspellen wanneer deze patiënt gedurende de tijd het meest kwetsbaar is. Als het lukt om een model met herhaalde bloedbiomarkers met een goede performance te ontwikkelen en te valideren, dan zou dit de manier waarop poliklinische patiënten worden behandeld kunnen veranderen. In een ideale

situatie zouden patiënten dan regelmatig bloed laten afnemen waarin de relevante biomarkers worden gemeten. Deze biomarkerconcentraties worden vervolgens ingevoerd in het voorspelmodel waarna na iedere bloedafname het nieuwe bijgewerkt risico op cardiale klachten in de komende periode wordt gegeven. Dit risico kan de cardioloog vervolgens gebruiken om preventief de medicatie aan te passen en zo ziekenhuisopname of andere klachten te voorkomen.
Bij HF-patiënten heeft onderzoek met herhaalde bloedbiomarkermetingen inderdaad veelbelovende resultaten opgeleverd voor risicovoorspelling. HF kan worden gekarakteriseerd als een proces van langdurige achteruitgang waarbij tal van pathofysiologische processen betrokken zijn. Het is bekend dat biomarkers die de toestand van deze processen weerspiegelen geassocieerd zijn met de huidige klinische status van de patiënt. Het bekendste voorbeeld van zo'n biomarker is NT-proBNP, dat in de klinische praktijk wordt gemeten om de ernst van decompensatie of de huidige HF-status te schatten. In het huidige proefschrift, maar ook in eerder werk van onze onderzoeksgroep, hebben we aangetoond dat het herhaaldelijk meten van biomarkersconcentraties tijdens de follow-up van HF-patiënten ons meer prognostische informatie geeft dan een enkele nulmeting. Voor verschillende biomarkers, waaronder bekende markers zoals NT-proBNP, ST2 en Cystatin C, is aangetoond dat het patroon tijdens de follow-up kan worden gebruikt om zowel hoogrisicopatiënten als hoogrisicoperiodes te voorspellen. Een toekomstige stap in deze onderzoekslijn zou kunnen zijn om te onderzoeken of het gebruik van een dergelijk HF-voorspelmodel in de klinische praktijk ook daadwerkelijk leidt tot goed getimede en juiste medicatieveranderingen en daarmee ziekenhuisopnames of andere behandelingen kunnen worden voorkomen. Hoewel veel invasiever en duurder dan herhaalde biomarkermetingen, heeft het CardioMEMS HF-systeem in zekere zin al de haalbaarheid en het potentieel van een dergelijke benadering aangetoond. CardioMEMS is een apparaat dat continu de pulmonale arteriële druk (PAP) meet. Een lagere PAP wordt geassocieerd met verbeterde klinische resultaten in patienten met chronisch HF. Met behulp van monitoring op afstand kan de cardioloog de huidige status van HF beoordelen en wanneer de PAP stijgt de behandeling wijzigen, ruim voordat de patiënt symptomen ervaart. In klinische onderzoeken heeft het CardioMEMS HF-systeem aangetoond dat het HF-ziekenhuisopnames vermindert en de kwaliteit van leven van de patiënten verbetert.

Bij patiënten met ACS zijn de resultaten van de voorspelmodellen met herhaalde metingen minder veelbelovend. Een ACS is een plotselinge manifestatie van het meer chronische proces van CAD. De pathofysiologie van het acute ACS is goed beschreven en omvat het scheuren van een bestaande plaque in de kransslagader, de vorming van een nieuw stolsels aldaar en tenslotte een occlusie. Ondanks de hoogfrequente bloedafname konden we in de BIOMArCS-study dit pathofysiologische proces niet goed vangen (en dus ook niet goed voorspellen). Geen één van de onderzochte biomarkers liet namelijk een duidelijke stijging zien voorafgaand aan het recidief ACS of aan de dood door cardiale oorzaak. In de studie konden verschillende biomarkers weliswaar hoog- en laagrisicopatiënten onderscheiden maar de toegevoegde prognostische waarde van de hoogfrequente bloedafname ten opzichte van een enkele nulmeting lijkt beperkt en weegt niet op tegen de extra inspanningen.

Ondanks dat de BIOMArCS-studie de timing van studie-eindpunt niet kon voorspellen, toonde het (opnieuw) aan dat biomarkers die de ernst van de onderliggende CAD weerspiegelen, kunnen worden gebruikt om hoogrisicopatiënten te identificeren tijdens de follow-up van 1 jaar. Het zou daarom interessant zijn om in een toekomstige studie te bekijken of laagfrequente biomarkermeting (bijvoorbeeld eens per zes maanden), zou kunnen bijdragen aan een continue risicobeoordeling van post-ACS-patiënten. Als dit het geval is, kunnen deze resultaten worden gebruikt om de behandeling van patiënten met chronische CAD te personaliseren.

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PhD Portfolio

	Year	Workload (ECTS)
NIHES Research Master	2015-2018	70
General PhD courses		
BROK cours	2016	1
Biomedical English Writing	2017	1.5
In-depth cardiovascular courses		
COEUR Cardiovascular imaging and diagnostics	2017	1.5
COEUR Heart failure research	2016	1.5
COEUR Intensive Care Part I	2017	0.5
Conferences		
AHA congress, New Orleans, United States of America	2016	1.5
ESC congress, Barcelona, Spain	2017	1.5
NHI translational Research Meeting, Utrecht, the Netherlands	2018	0.6
NVVC voorjaarscongres, Noordwijk aan Zee, the Netherlands	2018	0.6
Presentations		
AHA congress, New Orleans, United States of America	2016	0.3
ESC congress, Barcelona, Spain	2017	0.3
Biostatistic research meeting	2018	0.3
Teaching Activities		
Supervising medical students, *writing a systematic review*	2017	0.3
Supervising master thesis of a medical student	2017	1.5

About the author

Victor van den Berg was born on the 15th of May, 1987 in Hendrik-Ido-Ambacht, the Netherland,. After completing secondary school (Rotterdams Montessori Lyceum, Rotterdam) in 2005, he started medical school in Amsterdam at the University of Amsterdam. In 2007, Victor transferred to the medical school of the Erasmus University which he completed in the start of 2014. After obtaining his master degree, he worked as a resident (ANIOS) at the department of Internal Medicine and at the department of Cardiology at the Maasstad Ziekenhuis for 15 months.

In August 2015, Victor started his PhD project at the department of Cardiology of the Erasmus MC (Rotterdam) and the Northwest clinics (Alkmaar), supervised by prof. dr. Eric Boersma, dr. Isabella Kardys and dr. Victor Umans. He was primarily involved in data-analysis of prospective biomarker studies in patients with acute coronary syndromes and heart failure patients. While working as a PhD student, Victor also enrolled in the research master 'Clinical Epidemiology' of the Netherlands Institute for Health Sciences (NIHES), which he finished with honors in 2018.

Since October 2018 Victor is working as a resident in training, at the anesthesiology department of the Leiden University Medical Center.

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