
SYMBIOSIS

Part II

THE ROLE OF THE KIDNEYS IN HEART FAILURE AND BEYOND

CHAPTER 8

Temporal Patterns of 14 Blood Biomarkers of Cardiac Remodeling in Relation to Prognosis of Patients with Chronic Heart Failure – The Bio-SHiFT Study

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ABSTRACT

Background

Remodeling biomarkers carry high potential for predicting adverse events in chronic heart failure (CHF) patients. However, temporal patterns during the course of CHF, and especially the trajectory prior to an adverse event, are unknown. We studied the prognostic value of temporal patterns of 14 cardiac remodeling biomarker-candidates in stable patients with CHF.

Methods

In 263 CHF patients, we performed trimonthly blood sampling during a median follow-up of 2.2 years. For the analysis, we selected all baseline samples, the two samples closest to the primary endpoint (PE), or the last sample available for endpoint-free patients. Thus, in 567 samples, we measured ST2, Gal-3, Gal-4, GDF-15, MMP-2, 3 and 9, TIMP-4, PLC, AP-N, CASP3, CTSD, CTSZ and CSTB. The PE was a composite of cardiovascular mortality, heart transplantation, left ventricular assist device implantation and HF-hospitalization. Associations between repeatedly-measured biomarker-candidates and the PE were investigated by joint modelling.

Results

Median age was 68 (IQR:59-76) years with 72% men; 70 patients reached the PE. Repeatedly measured ST2, Gal-3, Gal-4, GDF-15, MMP-2 and 9, TIMP-4, PLC, CTSD and CSTB levels were strongly and significantly associated with the PE, and increased as the PE approached. The slopes of biomarker trajectories were also predictors of clinical outcome, independent of their absolute level. Associations persisted after adjustment for clinical characteristics and pharmacological treatment. ST2 was the strongest predictor (HR: 7.55 per SD difference, 95%CI: 5.53-10.30), followed by GDF-15 (4.06, 2.98-5.54) and MMP-2 (3.59, 2.55-5.05).

Conclusions

Temporal patterns of remodeling biomarker-candidates strongly predict adverse clinical outcomes in CHF.

INTRODUCTION

Chronic heart failure (CHF) is a complex syndrome that may result from a diverse spectrum of conditions preventing the left ventricle from properly filling and ejecting blood.[1] Beyond the traditional evaluation of suspected heart failure (HF) patients, the use of biomarkers is on the rise.[2] Circulating blood biomarkers are capable of detecting subtle changes in the pathophysiological processes underlying CHF, and can be measured with relative ease. Not only do they have a crucial role in the diagnosis of HF, but also in risk stratification of patients with CHF.

Since the introduction of natriuretic peptides, interest in other biomarkers has grown exponentially.[3] In this context, biomarkers of cardiac remodeling, which represent complex histological and structural myocardial changes, including cardiac hypertrophy, fibrosis and inflammation4, have recently gained wide attention. Consistent associations have been found between Suppression of tumorigenicity-2 (ST2), Galectin-3 (Gal-3) and Growth differentiation factor 15 (GDF-15) and adverse prognosis in CHF patients.[5-7] Overall, studies performed so far have shown that remodeling biomarkers carry high potential for predicting adverse events in CHF patients.[8]

Since blood biomarkers reflect the disease processes underlying CHF, their levels may be expected to change in accordance with disease severity, as well as prior to adverse events.[9] However, temporal patterns of remodeling biomarkers during the course of CHF, and especially temporal patterns shortly before an adverse event occurs have not yet been investigated in detail. Previous studies have mostly described the value of single, baseline measurements of cardiac remodeling biomarkers for prognosis. Only a few studies have been performed on serial assessment of, for example, ST2[10-12], but these studies were usually relatively small, or re-measured the biomarker during a brief first follow-up period only and then did not re-measure at regular intervals during longer-term follow-up. Furthermore, these studies have mostly used only one repeated measurement and described the change between two measurements, which does not properly capture the underlying temporal trajectory.[13]

Conversely, a recent report from the TRIUMPH study, which performed 7 repeated ST2 measurements during 1-year follow-up, clearly demonstrated the incremental value of temporal patterns derived from such frequent, repeated sampling in patients with acute HF[14], illustrating the need for further research on this topic. Accordingly, the aim of our study was to evaluate temporal patterns of 14 biomarker-candidates of cardiac remodeling and their value for predicting future adverse clinical events in patients with CHF. For this purpose, we performed repeated mea-

surements of the levels of ST2, Gal-3, Galectin-4 (Gal-4), GDF-15, extracellular matrix components, selected proteolytic enzymes and N-terminal pro–B-type natriuretic peptide (NT-proBNP) in 263 stable patients with CHF, and investigated the associations of these biomarker-candidate levels, and changes therein, with adverse clinical events.

METHODS

CHF cohort

The 'Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis' (BioSHiFT) study is a prospective cohort study of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Northwest Clinics, Alkmaar, The Netherlands.[15,16] Patients were included if aged ≥18 years, capable of understanding and signing informed consent, and if CHF had been diagnosed ≥3 months ago according to European Society of Cardiology guidelines.[17,18] Patients were ambulatory and stable, i.e., they had not been hospitalized for HF in the past three months. 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. This investigation comprised 263 CHF patients that were enrolled during the first inclusion period from October 2011 until June 2013. Follow-up lasted until 2015.

Study procedures

All patients were evaluated by research physicians, who collected information on HF-related symptoms, New York Heart Association (NYHA) class, and performed a physical examination. Information on HF etiology, left ventricular ejection fraction, cardiovascular risk factors, medical history and treatment was retrieved primarily from hospital records and was checked in case of ambiguities. History of cardiovascular and other comorbidities was defined as clinical diagnosis thereof reported in the hospital records. Glomerular filtration rate (GFR) was determined by the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation validated in HF patients.[19] Patients were categorized using National Kidney Foundation–Kidney Disease Outcome Quality Initiative (KDOQI) clinical practice guidelines.[20] Baseline NT-proBNP and Cardiac troponin T (hsTnT) were measured in 1 batch in stored serum samples as described before[15], using electrochemiluminescence immunoassays (Elecsys 2010; Roche Diagnostics, Indianapolis, IN).

All patients were followed at the outpatient clinic as part of standard care by their treating physicians, who were blinded for biomarker-candidate results. Additionally, study follow-up visits were predefined and scheduled every 3 months (±1 month). At each study follow-up visit, the research physician performed a short medical evaluation and blood samples were collected. During follow-up, all medication changes and occurrence of hospitalizations for HF, myocardial infarction (MI), percutaneous coronary intervention, coronary artery bypass grafting, arrhythmias, cerebrovascular accident, heart transplantation, left ventricular assist device (LVAD) implantation and mortality, were recorded in the electronic case report forms, and associated hospital records and discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker-candidate results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

The primary endpoint (PE) was a composite of cardiac death, heart transplantation, LVAD implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. We used the International Classification of Disease-10th revision (ICD-10), from the World Health Organization, to assign the endpoints.[21] Cardiac death was defined as death from MI or other ischemic heart disease (ICD-10 : codes I20-I25), death from other heart disease including HF (codes I30-I45 and I47-I52), sudden cardiac death (code I46), sudden death undefined (code R96) or unwitnessed or ill-described death (codes R98, R99). Hospitalization for acute or worsened HF was defined as a hospitalization for an exacerbation of HF symptoms, in combination with two of the following: 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.[17]

Laboratory procedures

Blood samples were collected at baseline and at each trimonthly study follow-up visit, and were processed and stored at -80^{o}C within two hours after collection. Treating physicians were unaware of biomarker-candidate results as these biomarker-candidates were measured batchwise after completion of follow-up. Thus, the biomarker-candidate measurements did not lead to drug adjustments. All patients received usual care. All laboratory personnel were blinded for clinical data and patient outcomes.

Selection of blood samples

Blood samples were drawn at each study follow-up visit, which were predefined and scheduled every 3 months (±1 month). Hence, in the first inclusion round of the Bio-SHiFT study which we used for the current investigation, we collected a total of 1984 samples before occurrence of the PE or censoring (9 (5–10) blood samples per patient). For reasons of efficiency, for the current investigation, we made a selection from these 1984 samples: we selected 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 PE (which, by design, were 3 months apart) (Figure 1). Our previous investigations in this cohort have demonstrated that several biomarker-candidates increase in the months prior to the incident adverse event.[15,16] Thus, by selecting the last 2 samples prior to the incident endpoint, we aimed to capture this increase. Conversely, in event-free patients, our previous investigations showed stable biomarker-candidate levels, in which case 1 additional sample suffices. Altogether, our selection amounted to 567 samples for the current analysis.

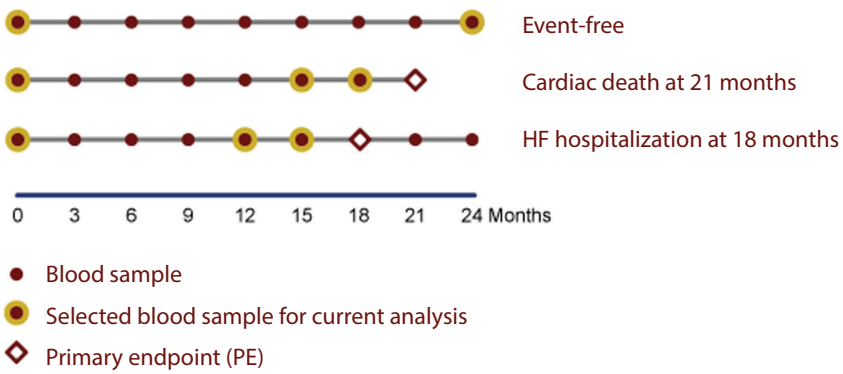


FIGURE 1 Selection of blood samples. At each study follow-up visit, the research physician performed a short medical evaluation and blood samples were collected. Study follow-up visit were predefined and scheduled every 3 months (±1 month). The primary endpoint (PE) was a composite of cardiac death, heart transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. For reasons of efficiency, for the current investigation we selected all baseline samples, the two samples closest in time prior to the PE, and the last sample available in patients in whom the PE did not occur during follow-up. Blood sampling continued after hospitalization, but since hospitalization for the management of acute or worsened HF was considered as PE, the two samples closest in time prior to hospitalization were selected for the current analysis.

Biomarker-candidate measurements

The Cardiovascular (CVD) panel III of the Olink Multiplex platform for new biomarkers (Olink Proteomics AB, Uppsala, Sweden) was used for analysis of high-abundance proteins. The proteins analyzed by the assay were chosen based on their potential to represent aspects of cardiovascular pathophysiology. The assay is based on proximity extension assay technology.[22] 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 polymerase chain reaction (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. In a validation study, the mean intra-assay and inter-assay coefficients of variation were 8% and 12%, respectively.[23] The biomarker-candidates are delivered in Normalized Protein Expression (NPX) Units, which are relative units that result from the PCR. They are expressed on a log2 scale where one unit higher NPX value 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. For the current investigation, ST2, Gal-3, Gal-4, GDF-15, matrix metalloproteinase (MMP)-2, 3 and 9, Tissue Inhibitor Metalloproteinase (TIMP)-4, Perlecan (PLC), aminopeptidase-N (AP-N), Caspase-3 (CASP3), Cathepsin D (CTSD), Cathepsin Z (CTSZ), Cystatin-B (CSTB) and NT-proBNP were examined.

Statistical analysis

Variables with a normal distribution are presented as mean ± standard deviation (SD), whereas the median and interquartile range (IQR) are presented in case of non-normality. Categorical variables are presented as counts and percentages. Freedom from composite endpoint was assessed using Kaplan-Meier analysis, first for the full cohort and then according to median biomarker-candidate value. Biomarker-candidates as measured by the Olink CVD III panel are presented as arbitrary, relative units (NPX values) on their linear scale (i.e., non-log transformed) in Table 1, Table 2 and Figure 3. In the below mentioned models, we used the Z-score (i.e., the standardized form) of the log2-transformed biomarkers to allow for direct comparisons of different biomarker-candidates.

We applied a joint modeling (JM) analysis to estimate the associations between patient-specific repeated biomarker-candidate levels and the hazard of the PE. JM combines linear mixed effect (LME) models for repeated measurements with Cox

proportional hazard models for the time-to-event data.[24] By doing this, all biomarker-candidate values were corrected for different follow-up durations between patients.[25] We studied the predictive value of absolute biomarker-candidate levels, as well as their rates of change (i.e., the slopes of the longitudinal biomarker trajectories).

In order to adjust for clinical risk determinants and potential confounders, we considered the following pre-defined models: 1) clinical model: LME and Cox models were adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and estimated glomerular filtration rate (eGFR); 2) clinical & time-varying HF medication model: additional adjustment for equivalent doses of carvedilol, enalapril, furosemide, and spironolactone (repeatedly assessed during follow-up) in a time-dependent Cox analysis; 3) established cardiac biomarker model: LME and Cox models were adjusted for the established biomarkers NT-proBNP and high-sensitive troponin T (hsTnT). Results are given as hazard ratios (HR) and 95% confidence intervals (CI) per 1SD difference of the absolute biomarker-candidate level and per 0.1 SD/year difference of the slope at any point in time during follow-up.

We examined a total of 15 serially measured blood biomarkers in relation to the PE (14 marker-candidates of remodeling, plus NT-proBNP). To correct for multiple testing, we performed matrix spectral decomposition.[26,27] Consequently, the corrected significance level was set at $p < 0.005$. We used the conventional $p < 0.05$ threshold to conclude significance for the relation between baseline characteristics and the occurrence of the PE (Table 1), as well as for the relation between first and last biomarker-candidate sample (Table 2). All tests were two-tailed. All analyses were performed with SPSS Statistics 24 (IBM Inc., Chicago, IL) and R Statistical Software using packages nlme[28] and JMbayes.[24] The matrix spectral decomposition application was available online.[29]

RESULTS

Baseline characteristics

Table 1 shows baseline characteristics in relation to the occurrence of the PE. Patients who experienced the PE during follow-up were older, had a lower systolic blood pressure, higher NYHA class and higher levels of NT-proBNP and hsTnT. Furthermore, they more frequently had diabetes and atrial fibrillation, and were more often on diuretics. The majority of the examined biomarker-candidates (ST2, Gal-3, Gal-4, GDF-15, MMP2, TIMP4, PLC, AP-N, CTSZ, CSTB and NTproBNP) showed significantly higher levels at baseline in patients who later experienced the endpoint than in patients who remained event-free (Table 1).

TABLE 1 **Patients characteristics in relation to the primary endpoint.**

Variable	Total	Composite endpoint reached		p-value*
		Yes	No	
n (%)	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, mm Hg	122 ± 20	117 ± 17	124 ± 21	0.020
Diastolic blood pressure, mm Hg	72 ± 11	70 ± 10	73 ± 11	0.055
Features of heart failure				
NYHA class III or IV	69 (26)	31 (44)	38 (20)	< 0.001
HFrEF	250 (95)	66 (94)	184 (95)	0.75
HFpEF	13 (5)	4 (6)	9 (5)	
LVEF	32 ± 10	30 ± 11	33 ± 10	0.18
Established biomarkers				
NT pro-BNP, 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
Etiology of heart failure, n (%)				
Ischemic	117 (44)	36 (51)	81 (42)	0.17
Hypertension	34 (13)	10 (14)	24 (12)	0.69
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, n (%)				
Prior MI	96 (37)	32 (46)	64 (33)	0.06
Prior PCI	82 (31)	27 (39)	55 (29)	0.12
Prior CABG	43 (16)	13 (19)	30 (16)	0.56
History of ICD implantation	156 (59)	44 (63)	112 (58)	0.48
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

continued

Variable	Total	Composite endpoint reached Yes	No	p-value*
Hypertension	120 (46)	38 (54)	82 (43)	0.090
COPD	31 (12)	12 (17)	19 (10)	0.11
Medication use, n (%)				
Beta-blocker	236 (90)	61 (87)	175 (91)	0.40
ACE-I or ARB	245 (93)	63 (90)	182 (94)	0.22
Diuretics	237 (90)	68 (97)	169 (88)	0.021
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
KDOQI classification, n (%)				
eGFR ≥ 90	28 (11)	7 (10)	21 (11)	0.18
eGFR 60-89	95 (36)	20 (28)	75 (39)	
eGFR 30-59	119 (45)	37 (53)	82 (42)	
eGFR < 30	21 (8)	6 (9)	15 (8)	
Biomarker level at baseline in arbitrary unit (linear NPX values)				
ST2	10.36 (7.25-13.65)	12.32 (8.41-17.20)	9.45 (7.05-12.23)	<0.001
Gal-3	38.47 (31.76-46.94)	42.60 (33.68-53.12)	38.20 (31.10-44.71)	0.007
Gal-4	8.90 (6.71-12.61)	12.32 (8.41-17.20)	9.45 (7.05-12.23)	0.001
GDF-15	45.23 (31.52-75.42)	66.01 (41.80-119.28)	41.38 (29.24-61.73)	<0.001
MMP-2	17.63 (14.03-22.67)	19.84 (15.28-27.47)	16.33 (13.09-21.56)	<0.001
MMP-3	76.13 (53.56-105.23)	77.24 (56.71-111.93)	76.10 (53.15-104.45)	0.31
MMP-9	9.10 (6.50-13.67)	9.54 (6.23-15.80)	8.69 (6.54-13.46)	0.45
TIMP4	17.14 (13.09-23.41)	20.89 (14.84-26.17)	16.24 (12.16-22.03)	<0.001
PLC	80.74 (60.76-110.60)	107.61 (73.44-145.58)	73.26 (57.79-98.69)	<0.001
AP-N	22.47 (18.73-28.59)	25.59 (18.68-32.44)	21.73 (18.69-27.28)	0.029
CASP3	262.88 (140.42-490.67)	295.91 (137.09-571.90)	257.34 (142.03-472.55)	0.32
CTSD	32.00 (25.47-41.42)	33.05 (27.18-46.44)	31.89 (24.98-41.05)	0.19
CTSZ	33.02 (26.16-44.45)	37.04 (26.65-49.51)	31.97 (25.9-42.65)	0.039
CSTB	51.12 (36.91-78.66)	76.85 (50.29-103.80)	46.77 (33.68-64.53)	<0.001
NT-proBNP	8.90 (3.82-16.93)	18.48 (11.19-33.71)	6.32 (2.82-12.39)	<0.001

MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; ACE-I, angiotensin-converting enzyme inhibitors; AP-N, aminopeptidase-N; ARB, angiotensin II receptor blockers; CASP3, Caspase-3(CASP3); CSTB, Cystatin-B; CTSD, Cathepsin D; CTSZ, Cathepsin Z; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; Gal-3, galectin-3; Gal-4, galectin-4; GDF-15, growth differentiation factor 15; ICD, Implantable Cardioverter Defibrillator; KDOQI, National Kidney Foundation–Kidney Disease Outcome Quality Initiative; NPX, normalized protein expression;

CVA, cerebrovascular accident; MMP-2, 3 and 9, matrix metalloproteinase 2, 3 and 9; NT-proBNP, N-terminal pro–B-type natriuretic peptide; NYHA class, New York Heart Association class; PLC, Perlecan; ST2, Suppression of tumorigenicity-2; TIA, transitory ischemic attack; TIMP-4, Tissue Inhibitor Metalloproteinase 4. Variables with a normal distribution are presented as mean ± SD, whereas non-normally distributed continuous variables are expressed as median (25^{th} – 75^{th} percentile). Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts, because of missing values. * p value <0.05. ‡ Cardiomyopathy comprised hypertrophic, dilated, restrictive, arrhytmogenic right ventricular, non-compaction cardiomyopathy or unclassified cardiomyopathy.

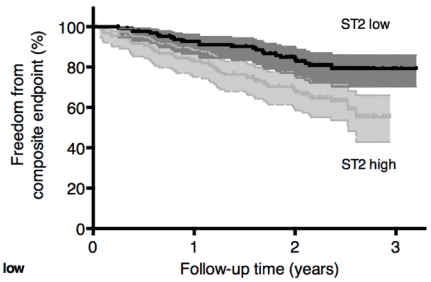
Follow-up and study endpoints

During a median (IQR) follow-up of 2.2 (1.4–2.5) years, we collected 9 (5–10) blood samples per patient. amounting to a total of 1984 samples. After selecting all baseline samples, the two samples closest in time to the composite endpoint, and the last sample available for event-free patients, 567 samples were available for the current investigation.

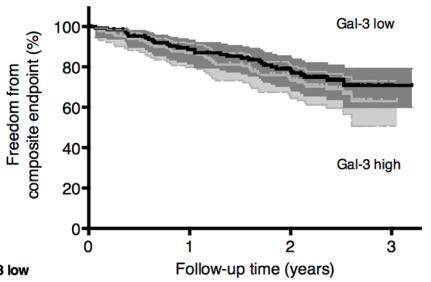
A total of 70 (27%) patients reached the PE: 56 patients were re-hospitalized for acute or worsened HF, 3 patients underwent heart transplantation, 2 patients underwent LVAD placement, and 9 patients died of cardiovascular causes. Overall, freedom from the composite endpoint was 0.76±0.03 at 2 years of follow-up (Figure S1). In particular baseline ST-2 and GDF-15 levels above the median showed worse freedom from composite endpoint (Figure 2).

Temporal patterns of biomarkers in relation to the occurrence of study endpoints

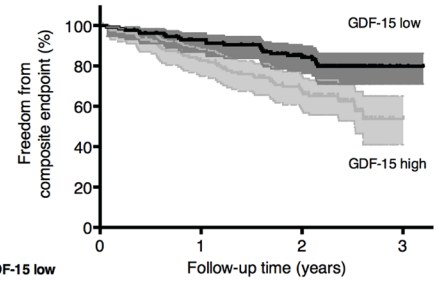
Figure 3 shows the average temporal patterns of cardiac remodeling biomarker-candidates in patients with and without the PE. Twenty-four months before occurrence of the endpoint, ST2 levels were already higher in patients who ultimately reached the PE compared to patients who remained event-free. Furthermore, ST2 significantly increased as the endpoint approached. All biomarker-candidates, except for CASP3 and CTSZ, showed a similar pattern although sometimes less outspoken.



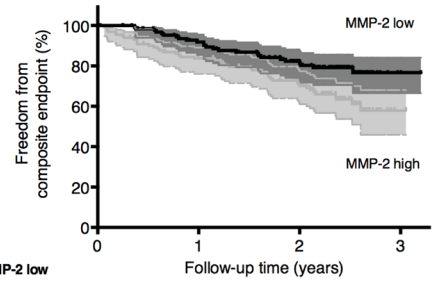
ST2 low				
Number of remaining patients:	130	111	85	54
Estimate:		0.93	0.84	0.79
St. Error:		± 0.02	± 0.03	± 0.04
ST2 high				
Number of remaining patients:	131	105	74	15
Estimate:		0.83	0.69	0.55
St. Error:		± 0.03	± 0.04	± 0.06



Gal-3 low				
Number of remaining patients:	130	105	79	27
Estimate:		0.89	0.78	0.71
St. Error:		± 0.03	± 0.04	± 0.05
Gal-3 high				
Number of remaining patients:	131	109	80	17
Estimate:		0.87	0.74	0.63
St. Error:		± 0.03	± 0.04	± 0.06



GDF-15 low				
Number of remaining patients:	130	114	86	72
Estimate:		0.93	0.84	0.80
St. Error:		± 0.02	± 0.03	± 0.04
GDF-15 high				
Number of remaining patients:	131	102	73	14
Estimate:		0.83	0.68	0.54
St. Error:		± 0.03	± 0.04	± 0.06



MMP-2 low				
Number of remaining patients:	130	109	80	30
Estimate:		0.92	0.81	0.77
St. Error:		± 0.03	± 0.04	± 0.05
MMP-2 high				
Number of remaining patients:	131	105	79	17
Estimate:		0.84	0.71	0.58
St. Error:		± 0.03	± 0.04	± 0.06

FIGURE 2 Freedom from the composite endpoint for ST2, Gal-3, GDF-15 and MMP-2 above and below the median value.

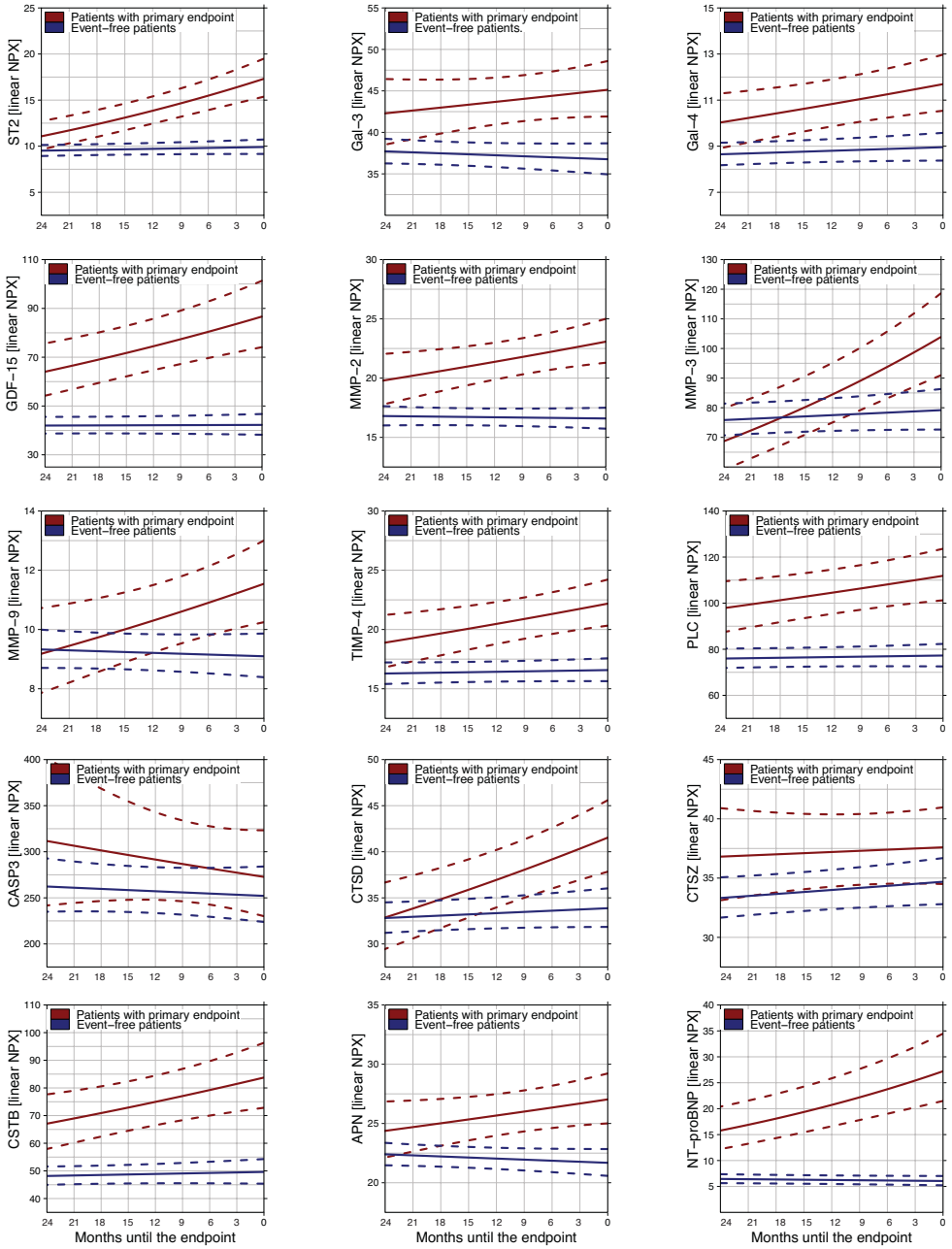


FIGURE 3 Average temporal patterns of cardiac remodeling biomarker-candidates during follow-up. X-axis: time remaining to the primary endpoint (for patients who experienced incident adverse events) or time remaining to last sample moment (for patients who remained event-free). Of note is that 'time zero' Is defined as the occurrence of the endpoint 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 on linear scale). Solid red line: Average temporal pattern of biomarker-candidate level in patients who reached the primary endpoint during follow-up. Solid blue line: Average temporal pattern of biomarker-candidate level in patients who remained endpoint free. Dashed lines: 95% confidence interval. AP-N, aminopeptidase-N; CASP3, Caspase-3; CSTB, Cystatin-B; CTSD, Cathepsin D; CTSZ, Cathepsin Z; Gal-3, galectin-3; Gal-4, galectin-4; GDF-15, growth differentiation factor 15; MMP-2, 3 and 9, matrix metalloproteinase 2, 3 and 9; NPX, Normalized Protein Expression; NT-proBNP, N-terminal pro–B-type natriuretic peptide; PLC, Perlecan; ST2, Suppression of tumorigenicity-2; TIMP-4, Tissue Inhibitor Metalloproteinase 4.

Table 3 shows the associations of cardiac remodeling biomarker-candidates with the PE. After adjustment for clinical characteristics, as well as after additional adjustment for HF medication doses during follow-up, ST2 was the numerically strongest predictor of the PE (HR 7.55 per 1 SD difference, 95%CI 5.53-10.30), followed by GDF-15 (HR 4.06, 95%CI 2.98-5.54) and MMP-2 (HR 3.59, 95%CI 2.55-5.05). Moreover, Gal-3, Gal-4, MMP-3 and 9, TIMP-4, PLC, AP-N, CTSD, CSTB, and NT-proBNP independently predicted the endpoint (all p-values <0.005). Furthermore, levels of these biomarker-candidates, except for MMP-3 and AP-N, remained significant predictors after adjusting for cardiac markers NT-proBNP and hsTnT.

Independently of their levels, the slopes (rates of change over time) of ST2, Gal-3, Gal-4, GDF-15, MMP-2, 3 and 9, TIMP-4, PLC, CTSD, and NT-proBNP remained significant predictors after adjusting for clinical characteristics and HF medication (clinical and time-varying medication model), as well as after adjustment for established cardiac biomarkers (established cardiac biomarker model, latter except for Gal-4 and MMP-3) (p-values <0.005, for HR see Table 4).

DISCUSSION

In this prospective repeated-measures study in 263 patients with stable CHF, we demonstrated that levels of biomarker-candidates of cardiac remodeling (such as ST2, Gal-3, Gal-4, GDF-15, MMP-2 and 9, TIMP-4, PLC, CTSD and CSTB) increase markedly and significantly as an adverse clinical event approaches. Importantly, their repeatedly measured levels strongly predict incident adverse clinical events with ST2 being the strongest predictor. Independently of their levels, the rate of biomarker change over time of these biomarker-candidates also predicts incident events. These associations persist after multivariable adjustment for clinical characteristics, pharmacological treatment during follow-up, and established cardiac biomarkers NT-proBNP and hsTnT.

TABLE 3 **Associations between blood biomarkers of cardiac remodeling and the primary endpoint – levels.**

	Crude model		Clinical model		Clinical and time-varying medication model		Cardiac biomarker model	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Level (per SD difference)								
ST2	5.63 (3.67-10.15)	<0.001*	5.93 (3.67-11.67)	<0.001*	7.55 (5.53-10.30)	<0.001*	4.02 (2.56-7.07)	<0.001*
Gal-3	1.91 (1.43-2.58)	<0.001*	2.11 (1.50-2.99)	<0.001*	3.23 (2.32-4.48)	<0.001*	1.57 (1.22-2.03)	<0.001*
Gal-4	1.92 (1.46-2.51)	<0.001*	1.68 (1.23-2.29)	<0.001*	2.11 (1.57-2.84)	<0.001*	1.54 (1.15-2.05)	<0.002*
GDF-15	3.09 (2.39-4.15)	<0.001*	3.11 (2.25-4.40)	<0.001*	4.06 (2.98-5.54)	<0.001*	2.50 (1.83-3.48)	<0.001*
MMP-2	3.17 (2.27-4.61)	<0.001*	3.21 (2.06-5.31)	<0.001*	3.59 (2.55-5.05)	<0.001*	2.45 (1.66–3.75)	<0.001*
MMP-3	1.60 (1.25-2.04)	0.001*	1.46 (1.08-1.95)	0.019	1.77 (1.35-2.32)	<0.001*	1.22 (0.93-1.61)	0.153
MMP-9	1.87 (1.32-2.69)	0.001*	1.75 (1.23-2.54)	<0.001*	2.53 (1.82-3.52)	<0.001*	1.75 (1.24-2.49)	<0.001*
TIMP-4	2.55 (1.83-3.61)	<0.001*	2.45 (1.65-3.81)	<0.001*	2.95 (2.13-4.09)	<0.001*	1.69 (1.21-2.40)	<0.001*
PLC	2.66 (1.98-3.60)	<0.001*	2.58 (1.76-3.88)	<0.001*	2.66 (1.96-3.62)	<0.001*	1.89 (1.32-2.73)	<0.001*
AP-N	2.04 (1.51-2.77)	<0.001*	1.75 (1.30-2.38)	<0.001*	1.83 (1.35-2.49)	<0.001*	1.53 (1.15-2.03)	0.005
CASP3	1.15 (0.83-1.58)	0.41	x		x		x	
CTSD	1.76 (1.37-2.28)	<0.001*	1.73 (1.26-2.35)	0.001*	1.80 (1.38-2.35)	<0.001*	1.67 (1.29-2.19)	<0.001*
CTSZ	1.37 (1.06-1.77)	0.023	x		x		x	
CSTB	2.10 (1.69-2.63)	<0.001*	2.39 (1.74-3.24)	<0.001*	2.93 (2.20-3.90)	<0.001*	1.70 (1.33-2.19)	<0.001*
NT-proBNP	4.50 (3.30-6.25)	<0.001*	4.35 (3.13-6.31)	<0.001*	4.80 (3.43-6.70)	<0.001*	4.27 (3.04-6.17)	<0.001*

HR (95% CI) are given per SD increase at any point in time during follow-up. **Crude model**: Cox model unadjusted, linear mixed effect (LME) model adjusted for sampling time; **Clinical model**: Cox and LME models adjusted for age, sex, diabetes, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, eGFR, and sampling time (LME); **Clinical and time-varying medication model**: Time-dependent Cox additionally adjusted for total daily doses carvedilol, enalapril, furosemide, and spironolactone during follow-up; **Cardiac biomarker model**: Cox and LME models adjusted for baseline NT-proBNP and hsTnT, and sampling time (LME). x not performed because repeatedly measured level was not significant; * p-value below the corrected significance level for multiple testing (p-value <0.005).

ST2 is an interleukin-1 receptor family member and an increase of the soluble circulating form of ST2 promotes myocardial apoptosis, fibrosis, and hypertrophy.[30] Higher ST2 plasma concentrations have shown to be among the strongest predictors of adverse outcome in CHF such as worsening HF and risk for either hospitalization or death from HF.[30] Accordingly, the updated guidelines for the management of HF suggest the use of ST2 for risk stratification in CHF patients.[31] In line with this, our study shows that ST2 is the biomarker-candidate whose association with adverse events is numerically the strongest out of the studied 14 biomarker-candidates of cardiac remodeling. Previously, several studies have examined the prognostic value of repeatedly measured ST2[10-12], but certain limitations restricted their generalizability. One study had a relatively short follow-up period of 10 months after recent HF decompensation[11], other studies re-measured ST2 infrequently (only in the beginning of the follow-up without regular measurements during the remaining follow-up), with clinical events occurring outside the sampling window.[10,12] Using such approaches, a relatively long time interval is left between the last ST2 measurement and the adverse event that occurs eventually. This may distort potential associations considering that CHF is a dynamic disease, and the levels of the biomarkers that reflect the underlying disease process may be expected to change as the adverse event approaches.[9] Ideally, the time interval between the last biomarker measurement and the adverse event should be kept as brief as possible in order to investigate accurately whether ST2 levels increase shortly before an adverse event and whether this increase truly contributes to the patient's risk. Another limitation is that the rate of change in ST2 might not be properly captured in former studies, as changes are often described as the difference between any two measurements without incorporating the time interval during which these changes occurred. In this way, the temporal biomarker pattern that occurs when an event is approaching is not taken into account, although this may in fact be of most value in individual risk prediction.

Our study extends current knowledge while addressing previous limitations, 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. This enabled us to select the two samples closest to an adverse event for our analyses. We show not only that ST2 levels differ at baseline between patients with and without incident events, but, importantly, we also demonstrate an increase in ST2 level as an adverse event approaches. Another unique finding is that the rate of the ST2 change over time independently predicts adverse clinical outcome. In other words, prognosis differs between patients who have high and stable ST2 levels and patients with high but rapidly increasing ST2 levels, which additionally underlines the incremental value of serial ST2 measurements.

TABLE 4 **Associations between blood biomarkers of cardiac remodeling and the primary endpoint – slopes.**

	Crude model		Clinical model		Clinical and time-varying medication model		Cardiac biomarker model	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Slope (per 0.1 SD/year difference)								
ST2	1.14 (1.08-1.21)	<0.001*	1.21 (1.11-1.34)	<0.001*	1.13 (1.11-1.16)	<0.001*	1.12 (1.06-1.18)	<0.001*
Gal-3	1.22 (1.11-1.36)	<0.001*	1.45 (1.19-1.88)	<0.001*	1.13 (1.07-1.19)	<0.001*	1.13 (1.05-1.22)	<0.001*
Gal-4	1.18 (1.05-1.36)	0.004*	1.27 (1.09-1.70)	<0.001*	1.10 (1.03-1.18)	<0.001*	1.12 (1.03-1.23)	0.015
GDF-15	1.22 (1.13-1.33)	<0.001*	1.29 (1.17-1.46)	<0.001*	1.12 (1.09-1.15)	<0.001*	1.18 (1.00-1.27)	<0.001*
MMP-2	1.19 (1.08-1.32)	<0.001*	1.29 (1.10-1.68)	<0.001*	1.18 (1.10-1.26)	<0.001*	1.14 (1.06-1.24)	<0.002*
MMP-3	1.16 (1.06-1.34)	0.002*	1.20 (1.04-1.57)	0.008	1.09 (1.04-1.15)	<0.001*	1.09 (1.02-1.19)	0.026
MMP-9	1.29 (1.19-1.41)	<0.001*	1.46 (1.27-1.76)	<0.001*	1.17 (1.12-1.23)	<0.001*	1.20 (1.12-1.29)	<0.001*
TIMP-4	1.31 (1.21-1.43)	<0.001*	1.03 (0.73-1.63)	0.25	1.16 (1.10-1.22)	<0.001*	1.23 (1.15-1.34)	<0.001*
PLC	1.26 (1.12-1.47)	<0.001*	1.37 (1.16-1.75)	<0.001*	1.13 (1.05-1.22)	0.002*	1.20 (1.08-1.33)	<0.001*
AP-N	1.16 (1.03-1.33)	0.010	x		x		x	
CASP3	0.84 (0.72-0.97)	0.018	x		x		x	
CTSD	1.27 (1.16-1.42)	<0.001*	1.47 (1.25-1.85)	<0.001*	1.15 (1.09-1.22)	<0.001*	1.16 (1.08-1.26)	<0.001*
CTSZ	1.21 (0.81-1.42)	0.16	x		x		x	
CSTB	1.00 (0.88-1.12)	0.97	x		x		x	
NT-proBNP	1.21 (1.10-1.36)	<0.001*	1.33 (1.15-1.63)	<0.001*	1.21 (1.16-1.27)	<0.001*	1.18 (1.08-1.30)	<0.001*

Hazard ratios (HRs) and 95% confidence intervals (CIs) are given per 0.1 SD of the annual slope at any point in time during follow-up. Annual slopes were additionally adjusted for the levels of repeatedly measured marker during follow-up. For adjusted models and abbreviations please see description under Table 3.

x not performed because repeatedly measured level was not significant; * p-value below the corrected significance level for multiple testing (p-value <0.005).

Gal-3 is a soluble β-galactoside-binding lectin and a member of the galectin family[32] and this biomarker is deemed a relevant mediator in the cardiac remodeling process.[33] A recent meta-analysis showed that increased Gal-3 levels carry higher risk of mortality independently of well-established risk factors.[34] Nevertheless, whether this association between Gal-3 and adverse outcome is independent of natriuretic peptides remained unclear.[35-37] In addition, studies on repeatedly measured Gal-3 are scarce. Our results show that repeatedly measured Gal-3 levels increase over time as an adverse event approaches, and that these levels significantly predict adverse clinical events even after multivariable adjustment that included NT-proBNP. These findings are also supported by Van der Velde et al., who showed that Gal-3 is of significant prognostic value in identifying high-risk CHF patients after combining data from the CORONA trial (baseline measurement plus additional measurement after 3 months) and the COACH trial (baseline measurement plus additional measurement after 6 months).[9] Less is known about Gal-4, another member of the galectin family. Although its physiological and pathophysiological features still need clarification, our results suggest that Gal-4 might be a promising biomarker in CHF patients since its level, as well as its change over time, showed a strong association with the PE.

In pathological conditions, GDF-15, a remote member of the transforming growth factor-β (TGF-β) super family, may influence cardiac remodeling via two different mechanisms, i.e., protection from apoptosis and induction of hypertrophy.[38] Several studies have shown promising results on the prognostic value of GDF-15. Chan et al.[39] found prognostic utility of GDF-15 measured at 6 weeks and 5 months beyond NT-proBNP in both HF patients with reduced ejection fraction (HFrEF) and those with preserved ejection fraction (HFpEF). In the HF-ACTION Study (HFrEF patients)[7], GDF-15 provided independent prognostic information incremental to hsTnT and NT-proBNP. Our results support and extend these findings by demonstrating that repeatedly measured levels of GDF-15, together with ST2, MMP-2 and NT-proBNP, show the numerically strongest independent associations with the PE (also after multivariable adjustment).

Biomarkers of cardiac extracellular matrix turnover include MMPs, their inhibitors (TIMPs), and the less studied PLC and AP-N. Several MMPs and TIMPs are associated with fibrosis, diastolic dysfunction and left ventricular hypertrophy[40,41], and some of these, such as MMP-9 and TIMP-1, correlated with the severity of CHF[42]. Moreover MMPs are implicated in several cardiovascular diseases; for example MMP-2 and -9 are potential biomarkers of acute myocardial infarction43 and coronary artery disease.[44] Furthermore, MMP-2 may be most suitable for serial biomarker measurements, as suggested by Täger et al. who performed

multiple measurements over a time span of 2 weeks of MMP-2, MMP-9, TIMP-1, and TIMP-4 in 50 patients with CHF.[45] In our study MMP-2, MMP-9, TIMP-4 and PLC were clear predictors of the PE. Conversely, level and slope of MMP-3 was not a significant predictor of adverse events after adjustment. AP-N is a type II metalloprotease[46], which is relatively unknown in the field of cardiac diseases. Although AP-N level was a strong predictor of the PE in our study, the rate of change over time (i.e., slope) was not. These results suggest that repeated measurement of AP-N may be unnecessary for prognostication, and single measurement may suffice.

Little or no data is available on biomarkers of apoptosis, like CASP3, CTSD, CTSZ and CSTB, and their role in cardiac remodeling and CHF prognosis. However, apoptosis has been investigated as a pathophysiologic mechanism in CHF. Since this study demonstrates interesting results regarding the prognostic value of the level of CSTB and both level and slope of CTSD, further investigations of the role of these novel biomarker-candidates in CHF should be encouraged.

Of interest, patients in the current study were in a better health condition than previously reported CHF populations since 74% was in NYHA class I-II. Still, we were able to show that biomarker-candidates of cardiac remodeling are strongly associated with clinical outcome. These findings raise the hypothesis that this NYHA class I-II patient group in particular may benefit from serial measurements of the studied biomarkers for prognostication, and ultimately to guide therapeutic interventions in order to prevent progression to advanced stage disease.

Study limitations

Our study carries several limitations. Firstly, as described before[16], our cohort comprised mainly HFrEF patients. This can most likely be attributed to the fact that in the Netherlands, most HFpEF patients are followed in secondary referral centers or by the general practitioner, while the current study was performed in two tertiary referral centers. Secondly, although we had trimonthly blood samples available for all patients, because of efficiency reasons 2 sampling moments were selected for event-free patients, and 3 sampling moments for patients with a PE. In previous investigations in this cohort[15], we have used all available sampling moments to determine NT-proBNP, hsTnT, C-reactive protein (CRP) as well as glomerular and tubular renal biomarkers.[16] Those investigations demonstrated that most of these biomarker-candidates show an increase shortly prior to the incident adverse event. Thus, we believe that by selecting baseline samples, as well as the last 2 samples prior to the incident endpoint, we retain the most informative measurements while enhancing efficiency. Finally, the assay we used for measuring the biomarker-can-

didates was designed as a biomarker discovery tool rather than being an approved clinical test. Future research should investigate standardization of the assays in order to successfully translate these emerging biomarkers into daily clinical practice.

CONCLUSION

This study shows that temporal patterns of patient-specific levels of numerous biomarker-candidates of cardiac remodeling strongly predict clinical outcome in CHF; specifically, these remodeling biomarker-candidates increase prior to an adverse event in CHF patients. These patient-specific temporal patterns indicate a promising role of these biomarker-candidates for individual prognostication and treatment monitoring.

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SUPPLEMENTARY INFORMATION

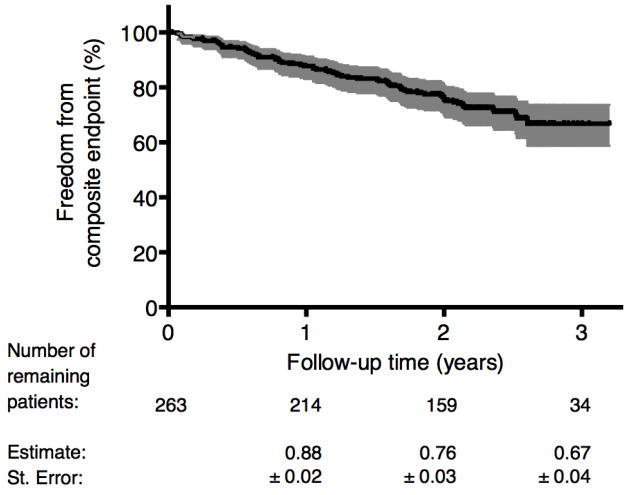

FIGURE S1 Freedom from the composite endpoint.