

# Temporal Patterns of 14 Blood Biomarker candidates of Cardiac Remodeling in Relation to Prognosis of Patients With Chronic Heart Failure—The Bio-SHiFT Study

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**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 before an adverse event, are unknown. We studied the prognostic value of temporal patterns of 14 cardiac remodeling biomarker candidates in stable patients with CHF from the Bio-SHiFT (Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis) study.

**Methods and Results**—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 2 samples closest to the primary end point (PE), or the last sample available for end point-free patients. Thus, in 567 samples, we measured suppression of tumorigenicity-2, galectin-3, galectin-4, growth differentiation factor-15, matrix metalloproteinase-2, 3, and 9, tissue inhibitor metalloproteinase-4, perlecan, aminopeptidase-N, caspase-3, cathepsin-D, cathepsin-Z, and cystatin-B. 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 modeling. Median age was 68 (interquartile range: 59–76) years with 72% men; 70 patients reached the PE. Repeatedly measured suppression of tumorigenicity-2, galectin-3, galectin-4, growth differentiation factor-15, matrix metalloproteinase-2 and 9, tissue inhibitor metalloproteinase-4, perlecan, cathepsin-D, and cystatin-B levels were 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. Suppression of tumorigenicity-2 was the strongest predictor (hazard ratio: 7.55 per SD difference, 95% CI: 5.53–10.30), followed by growth differentiation factor-15 (4.06, 2.98–5.54) and matrix metalloproteinase-2 (3.59, 2.55–5.05).

**Conclusions**—Temporal patterns of remodeling biomarker candidates predict adverse clinical outcomes in CHF.

**Clinical Trial Registration**—URL: <http://www.clinicaltrials.gov>. Unique identifier: NCT01851538. (*J Am Heart Assoc.* 2019;8:e009555. DOI: 10.1161/JAHA.118.009555.)

**Key Words:** biomarkers • cardiac remodeling • heart failure • prognosis • repeated measurements

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.<sup>1</sup> Beyond the traditional evaluation of suspected heart

failure (HF) patients, the use of biomarkers is on the rise.<sup>2</sup> 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

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Accompanying Figures S1 through S3 are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.118.009555>

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Received April 24, 2018; accepted January 3, 2019.

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## Clinical Perspective

### What Is New?

- We demonstrate explicitly that in chronic heart failure patients, biomarker candidates of cardiac remodeling increase before the occurrence of adverse events, and that their temporal patterns are associated with hospitalization for decompensated heart failure and cardiac mortality.

### What Are the Clinical Implications?

- These findings suggest a promising role for these biomarker candidates for individual risk profiling, and therefore future studies should assess the utility of serial measurements of cardiac remodeling biomarkers for guiding therapeutic interventions during management of chronic heart failure patients.

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.<sup>3</sup> In this context, biomarkers of cardiac remodeling, which represent complex histological and structural myocardial changes, including cardiac hypertrophy, fibrosis, and inflammation,<sup>4</sup> 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.<sup>5–7</sup> Overall, studies performed so far have shown that remodeling biomarkers carry high potential for predicting adverse events in CHF patients.<sup>8</sup>

Since blood biomarkers reflect the disease processes underlying CHF, their levels may be expected to change in accordance with disease severity, as well as before adverse events.<sup>9</sup> 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,<sup>10–12</sup> 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 1 repeated measurement and described the change between 2 measurements, which does not properly capture the underlying temporal trajectory.<sup>13</sup>

Conversely, a recent report from the TRIUMPH (Translational Initiative on Unique and novel strategies for

Management of Patients with Heart failure) 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,<sup>14</sup> 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 measurements 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 Bio-SHiFT (Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis) study is a prospective cohort study of stable patients with CHF, conducted in Erasmus MC, Rotterdam, and Northwest Clinics, Alkmaar, The Netherlands.<sup>15,16</sup> Patients were included if aged  $\geq 18$  years, capable of understanding and signing informed consent, and if CHF had been diagnosed  $\geq 3$  months ago according to European Society of Cardiology guidelines.<sup>17,18</sup> Detailed inclusion and exclusion criteria are shown in Figure S1. Patients were ambulatory and stable (ie, they had not been hospitalized for HF in the past 3 months). The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki, and registered in ClinicalTrials.gov (NCT01851538). The data that support the findings of this study will be made available to other researchers for purposes of reproducing the results upon reasonable request and in accordance with a data-sharing agreement. Written informed consent was obtained from all patients. This investigation comprised 263 CHF patients who 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 cause, 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 was determined by the Chronic Kidney Disease–Epidemiology Collaboration equation validated in HF patients.<sup>19</sup> Patients were categorized using National Kidney Foundation–Kidney Disease Outcome Quality Initiative clinical practice guidelines.<sup>20</sup> Baseline NT-proBNP and highly sensitive cardiac troponin T (hsTnT) were measured in 1 batch in stored serum samples as described before,<sup>15</sup> 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 to biomarker candidate results. Additionally, study follow-up visits were predefined and scheduled every 3 months ( $\pm 1$  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, percutaneous coronary intervention, coronary artery bypass grafting, arrhythmias, cerebrovascular accident, heart transplantation, left ventricular assist device 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 end points.

The primary end point (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. We used the *International Classification of Disease-10th revision (ICD-10)*, from the World Health Organization, to assign the end points.<sup>21</sup> Cardiac death was defined as death from myocardial infarction 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 2 of the following: BNP or NT-proBNP  $>3\times$  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.<sup>17</sup>

## Laboratory Procedures

Blood samples were collected at baseline and at each trimonthly study follow-up visit, and were processed and stored at  $-80^{\circ}\text{C}$  within 2 hours after collection. Treating

physicians were unaware of biomarker candidate results because these biomarker candidates were measured batch-wise 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 to 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 ( $\pm 1$  month). Hence, in the first inclusion round of the Bio-SHiFT study that 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 2 samples available closest in time before the PE (which, by design, were 3 months apart) (Figure S2). Our previous investigations in this cohort have demonstrated that several biomarker candidates increase in the months before the incident adverse event.<sup>15,16</sup> Thus, by selecting the last 2 samples before the incident end point, 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.

## 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. This platform enables simultaneous measurement of multiple proteins in 1 plasma sample, a principle common to several other multiplexing techniques. The proteins analyzed by the assay were chosen based on their potential to represent aspects of cardiovascular pathophysiology. A unique feature of this particular multiplexing assay is that it is based on proximity extension assay technology.<sup>22</sup> In brief, the assay uses 2 oligonucleotide-labeled antibodies to bind to their respective target proteins in the sample. When the 2 antibodies are in close proximity, a new polymerase chain reaction target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using standard real-time polymerase chain reaction. Four internal controls and 2 external controls were included in each assay. In a validation study, the mean intra-assay and interassay coefficients of variation were 8% and 12%, respectively.<sup>23</sup> The biomarker candidates are delivered in Normalized Protein Expression

**Table 1.** Baseline Characteristics in Relation to the Occurrence of the Primary End Point During Follow-Up

Variable	Total	Composite End Point Reached		P Value*
		Yes	No	
N (%)	263 (100)	70 (27)	193 (73)	
Demographics				
Age, y	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 <sup>2</sup>	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
Heart failure with reduced ejection fraction	250 (95)	66 (94)	184 (95)	0.75
Heart failure with preserved ejection fraction	13 (5)	4 (6)	9 (5)	
Left ventricular ejection fraction	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 1.73 m <sup>2</sup>	58 (43–76)	53 (40–73)	59 (44–77)	0.20
Cause of heart failure, n (%)				
Ischemic	117 (44)	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 <sup>‡</sup>	68 (26)	15 (21)	53 (28)	0.32
Unknown or others	32 (12)	4 (6)	28 (15)	
Medical history, n (%)				
Prior myocardial infarction	96 (37)	32 (46)	64 (33)	0.06
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
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
Hypertension	120 (46)	38 (54)	82 (43)	0.090
COPD	31 (12)	12 (17)	19 (10)	0.11
Medication use, n (%)				
β-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

Continued

Table 1. Continued

Variable	Total	Composite End Point Reached		P Value*
		Yes	No	
N (%)	263 (100)	70 (27)	193 (73)	
Thiazides	7 (3)	3 (4)	4 (2)	0.39
Aldosterone antagonist	179 (68)	53 (76)	126 (65)	0.11
KDOQI classification, n (%)				
eGFR $\geq 90$ mL/min per 1.73 m <sup>2</sup>	28 (11)	7 (10)	21 (11)	0.18
eGFR 60–89 mL/min per 1.73 m <sup>2</sup>	95 (36)	20 (28)	75 (39)	
eGFR 30–59 mL/min per 1.73 m <sup>2</sup>	119 (45)	37 (53)	82 (42)	
eGFR $< 30$ mL/min per 1.73 m <sup>2</sup>	21 (8)	6 (9)	15 (8)	

Variables with a normal distribution are presented as mean $\pm$ SD, whereas non-normally distributed continuous variables are expressed as median (25th–75th percentile). Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts, because of missing values. Missing values  $< 5\%$ , except for systolic blood pressure (5.3%). ACE-I indicates angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident; eGFR, estimated glomerular filtration rate; HsTnT, highly sensitive cardiac troponin T; ICD, implantable cardioverter defibrillator; KDOQI, National Kidney Foundation–Kidney Disease Outcome Quality Initiative; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA class, New York Heart Association class; PLC, perlecan; TIA, transient ischemic attack.

\* $P < 0.05$ .

†Cardiomyopathy comprised hypertrophic, dilated, restrictive, arrhythmogenic right ventricular, noncompaction cardiomyopathy or unclassified cardiomyopathy.

Units, which are relative units that result from the polymerase chain reaction. They are expressed on a log<sub>2</sub> scale where 1 unit higher Normalized Protein Expression 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, aminopeptidase-N (AP-N), caspase-3, cathepsin D (CTSD), cathepsin Z, cystatin-B (CSTB), and NT-proBNP were examined.

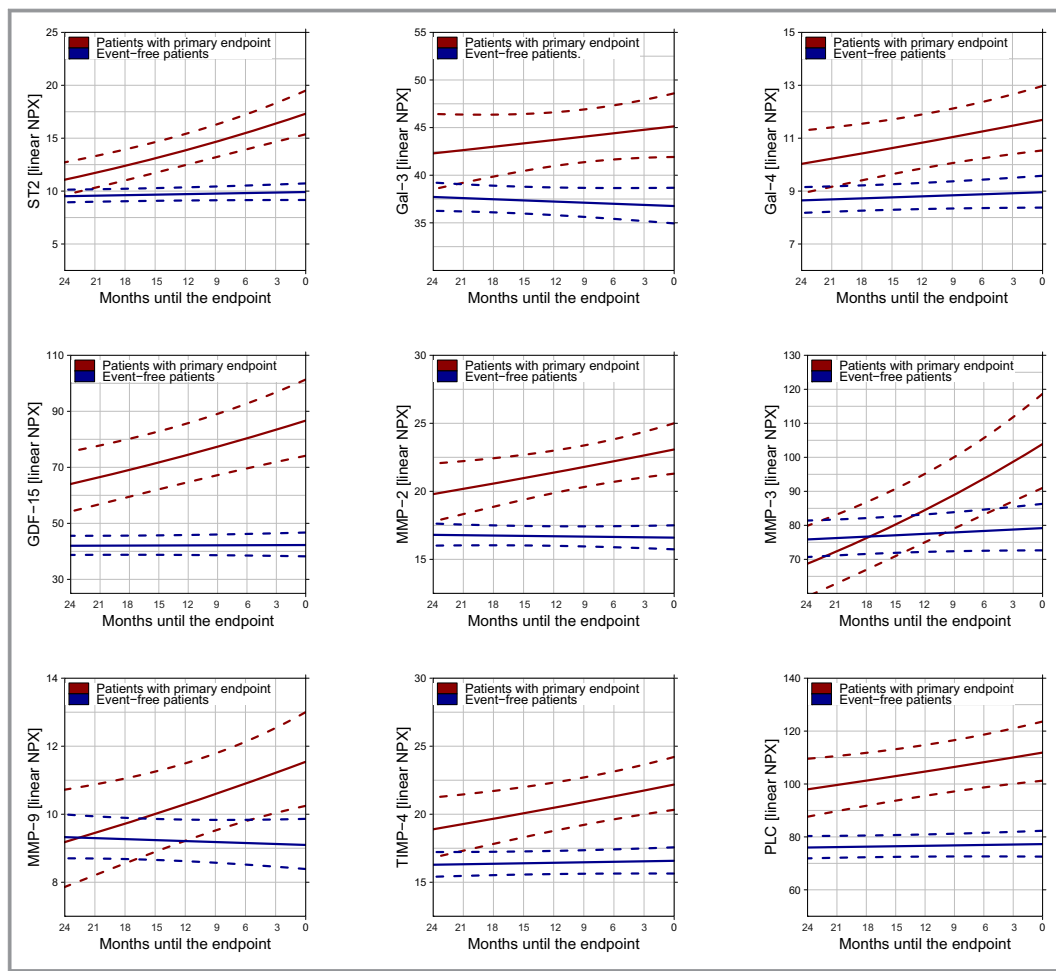
## Statistical Analysis

Variables with a normal distribution are presented as mean $\pm$ SD, whereas the median and interquartile range are presented in case of non-normality. Categorical variables are presented as counts and percentages (Table 1). Freedom from composite end point was assessed using Kaplan–Meier analysis, first for the full cohort and then according to median biomarker candidate value. Groups were compared by means of the log-rank test. To plot the average temporal patterns of cardiac remodeling biomarker candidates in patients with and without the PE, we used linear mixed effect models (as presented in Figure). Biomarker candidates as measured by the Olink CVD III panel are presented in arbitrary, relative units (Normalized Protein Expression values) on a linear scale (ie, non-log transformed) in Table 2 and Figure. In further analyses, we used the Z-score (ie, the standardized form) of the log<sub>2</sub>-transformed biomarkers to allow for direct comparisons of different biomarker candidates.

We applied a joint modeling analysis to estimate the associations between patient-specific repeated biomarker candidate levels and the hazard of the PE. Joint modeling combines linear mixed effect models for temporal evolution of the repeated measurements with Cox proportional hazard models for the time-to-event data.<sup>24</sup> By applying joint modeling, all biomarker candidate values were inherently corrected for different follow-up durations between patients.<sup>25</sup> We studied the predictive value of biomarker candidate levels, as well as their rates of change (ie, the slopes of the longitudinal biomarker trajectories). The latter analysis is of particular interest in situations where, for example, at a specific time point 2 patients show similar marker levels, but different rate of change of the marker.<sup>26</sup>

In order to adjust for clinical risk determinants and potential confounders, we considered the following predefined models: (1) clinical model: linear mixed effect and Cox models were adjusted for age, sex, diabetes mellitus, atrial fibrillation, baseline NYHA class, diuretics, systolic blood pressure, and estimated glomerular filtration rate; (2) clinical and 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; and (3) established cardiac biomarker model: linear mixed effect and Cox models were adjusted for the established biomarkers NT-proBNP and hsTnT. Results are given as hazard ratios (HR) and 95% CI per 1 SD difference of the absolute biomarker candidate level and per 0.1 SD/y difference of the slope at any point in time during follow-up.





**Figure.** Average temporal patterns of cardiac remodeling biomarker candidates during follow-up. X-axis: time remaining to the primary end point (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 end point and is depicted on the right side of the x-axis, so that the average marker trajectory can be visualized as the end point approaches (inherently to this representation, baseline sampling occurred before “time zero”). 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 end point during follow-up. Solid blue line: Average temporal pattern of biomarker candidate level in patients who remained end point free (solid blue line). Dashed lines: 95% CI. AP-N indicates 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.

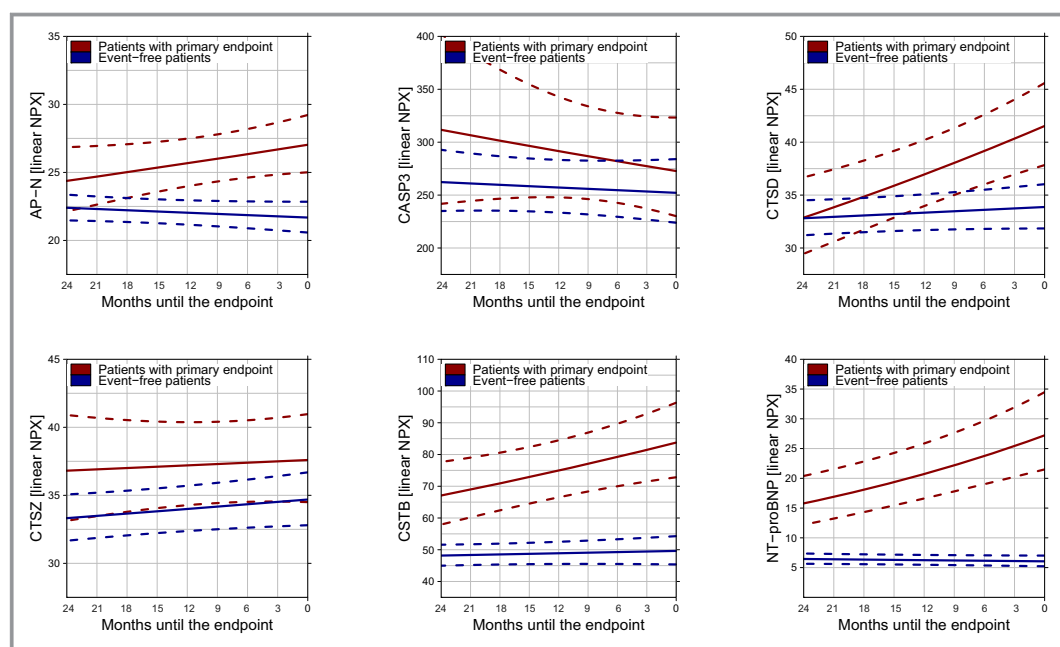
We examined a total of 15 serially measured blood biomarkers in relation to the PE (14 marker candidates of remodeling, plus NT-proBNP). To correct for multiple testing, we performed matrix spectral decomposition.<sup>27,28</sup> Consequently, the corrected significance level was set at  $P < 0.005$  (Tables 2 through 4 and Figure S3). We used the conventional  $P < 0.05$  threshold to conclude significance for the relation between baseline characteristics and the occurrence of the PE during follow-up (Table 1). All tests were 2-tailed. All analyses were performed with SPSS Statistics 24 (IBM Inc,

Chicago, IL) and R Statistical Software using packages nlme<sup>29</sup> and JMBayes.<sup>24</sup> The matrix spectral decomposition application was available online.<sup>30</sup>

## Results

### Baseline Characteristics

Table 1 shows baseline characteristics in relation to the occurrence of the PE. Patients who experienced the PE during



**Figure.** Continued.

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 mellitus 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, perlecan, AP-N, cathepsin Z, cystatin-B, and NTproBNP) showed significantly higher levels at baseline in patients who later experienced the end point than in patients who remained event-free (Table 2).

### Follow-Up and Study End Points

During a median (interquartile range) follow-up of 2.2 (1.4–2.5) years, a total of 70 (27%) patients reached the PE: 56 patients were rehospitalized for acute or worsened HF, 3 patients underwent heart transplantation, 2 patients underwent left ventricular assist device placement, and 9 patients died of cardiovascular causes. After selecting all baseline samples, the 2 samples closest in time to the composite end point, and the last sample available for event-free patients, 567 samples were available for the current investigation as described before (Figure S2).

### Median Marker Concentrations

Table 2 shows the median concentrations of biomarker candidates at all available measurement moments used for the current analysis. Overall, for several biomarker candidates, differences in level are present between the baseline

samples and the last samples available in patients who reached the composite end point, while in those that remained end point-free differences are less pronounced. For example, median concentrations of ST2 are already significantly different at baseline between patients who will reach the composite end point versus patients who will remain end point free. Furthermore, comparing the baseline sample and last sample, there is an increase of ST2 from baseline (12.32 [8.41–17.20] linear Normalized Protein Expression) to the second-last sample (15.10 [9.30–23.34]) and the last sample before the event (18.58 [10.27–28.32]), while in those who remained end point-free the difference is less pronounced (9.45 [7.05–12.23] at baseline versus 10.04 [7.39–13.25] at last sample).

Overall, freedom from the composite end point was  $0.76 \pm 0.03$  at 2 years of follow-up. In particular, baseline ST-2, Gal-4, GDF-15, perlecan, cystatin-B, and NT-proBNP levels above the median showed worse freedom from composite end point (Figure S3) (all with  $P < 0.005$ ).

### Temporal Patterns of Biomarkers in Relation to the Occurrence of Study End Points

Figure shows the average temporal patterns of cardiac remodeling biomarker candidates in patients with and without the PE. Twenty-four months before occurrence of the end point, ST2 levels were already higher in patients who ultimately reached the PE compared with patients who remained event free (“time zero” is defined as the occurrence

**Table 2.** Median Concentrations of Biomarker Candidates in Baseline Sample, Second-Last Sample, and Last Sample Available

Moment of Sampling	Biomarker Candidate	Total 263 (100)	Composite End Point Reached		P Value*
			Yes 70 (27)	No 193 (73)	
			Biomarker level in arbitrary unit (linear NPX values) <sup>†</sup>		
Baseline sample	ST2	10.36 (7.25–13.65)	12.32 (8.41–17.20)	9.45 (7.05–12.23)	<0.001*
Second-last sample <sup>‡</sup>			15.10 (9.30–23.34)		
Last sample <sup>§</sup>		10.51 (7.65–15.48)	18.58 (10.27–28.32)	10.04 (7.39–13.25)	<0.001*
Baseline sample	GAL-3	38.47 (31.76–46.94)	42.60 (33.68–53.12)	38.20 (31.10–44.71)	0.007
Second-last sample <sup>‡</sup>			44.05 (34.68–55.37)		
Last sample <sup>§</sup>		37.78 (31.53–46.84)	46.46 (36.06–58.57)	36.28 (30.33–44.59)	<0.001*
Baseline sample	GAL-4	8.90 (6.71–12.61)	12.32 (8.41–17.20)	9.45 (7.05–12.23)	0.001*
Second-last sample <sup>‡</sup>			11.75 (8.55–14.41)		
Last sample <sup>§</sup>		9.41 (6.75–13.30)	12.20 (9.34–16.09)	8.65 (6.58–12.40)	<0.001*
Baseline sample	GDF-15	45.23 (31.52–75.42)	66.01 (41.80–119.28)	41.38 (29.24–61.73)	<0.001*
Second-last sample <sup>‡</sup>			77.95 (46.83–122.06)		
Last sample <sup>§</sup>		42.72 (31.71–84.55)	94.88 (57.35–133.99)	39.34 (28.31–63.41)	<0.001*
Baseline sample	MMP-2	17.63 (14.03–22.67)	19.84 (15.28–27.47)	16.33 (13.09–21.56)	<0.001*
Second-last sample <sup>‡</sup>			21.57 (17.18–28.32)		
Last sample <sup>§</sup>		18.04 (14.06–22.23)	23.14 (18.33–28.40)	16.68 (13.68–20.80)	<0.001*
Baseline sample	MMP-3	76.13 (53.56–105.23)	77.24 (56.71–111.93)	76.10 (53.15–104.45)	0.31
Second-last sample <sup>‡</sup>			88.92 (59.61–126.78)		
Last sample <sup>§</sup>		86.01 (55.84–115.58)	97.13 (64.77–164.75)	81.03 (55.30–112.65)	0.013
Baseline sample	MMP-9	9.10 (6.50–13.67)	9.54 (6.23–15.80)	8.69 (6.54–13.46)	0.45
Second-last sample <sup>‡</sup>			10.11 (7.41–15.84)		
Last sample <sup>§</sup>		9.50 (6.87–13.34)	11.03 (8.31–15.20)	9.01 (6.57–13.05)	0.030
Baseline sample	TIMP4	17.14 (13.09–23.41)	20.89 (14.84–26.17)	16.24 (12.16–22.03)	<0.001*
Second-last sample <sup>‡</sup>			21.57 (16.34–27.05)		
Last sample <sup>§</sup>		17.32 (13.47–24.46)	24.63 (18.02–29.03)	16.12 (12.68–21.67)	<0.001*
Baseline sample	PLC	80.74 (60.76–110.60)	107.61 (73.44–145.58)	73.26 (57.79–98.69)	<0.001*
Second-last sample <sup>‡</sup>			111.79 (79.55–146.07)		
Last sample <sup>§</sup>		81.61 (62.41–117.27)	117.27 (90.68–147.45)	73.90 (60.18–104.86)	<0.001*
Baseline sample	AP-N	22.47 (18.73–28.59)	25.59 (18.68–32.44)	21.73 (18.69–27.28)	0.029
Second-last sample <sup>‡</sup>			25.63 (19.01–33.09)		
Last sample <sup>§</sup>		22.45 (18.56–28.16)	26.73 (20.78–35.04)	21.83 (18.24–25.99)	<0.001*
Baseline sample	CASP3	262.88 (140.42–490.67)	295.91 (137.09–571.90)	257.34 (142.03–472.55)	0.32
Second-last sample <sup>‡</sup>			284.27 (149.98–515.50)		
Last sample <sup>§</sup>		231.86 (141.08–425.87)	246.72 (141.29–477.90)	227.89 (140.98–416.30)	0.96
Baseline sample	CTSD	32.00 (25.47–41.42)	33.05 (27.18–46.44)	31.89 (24.98–41.05)	0.19
Second-last sample <sup>‡</sup>			37.75 (29.10–49.37)		
Last sample <sup>§</sup>		33.79 (27.06–45.05)	42.09 (32.30–52.41)	31.65 (26.45–41.81)	<0.001*
Baseline sample	CTSZ	33.02 (26.16–44.45)	37.04 (26.65–49.51)	31.97 (25.90–42.65)	0.039
Second-last sample <sup>‡</sup>			37.02 (29.06–48.39)		
Last sample <sup>§</sup>		35.46 (27.88–43.88)	39.44 (30.97–49.88)	34.43 (27.00–42.44)	0.045

Continued



Table 2. Continued

Moment of Sampling	Biomarker Candidate	Total	Composite End Point Reached		P Value*
			Yes	No	
		263 (100)	70 (27)	193 (73)	
Baseline sample	CSTB	51.12 (36.91–78.66)	76.85 (50.29–103.80)	46.77 (33.68–64.53)	<0.001*
Second-last sample <sup>‡</sup>			75.77 (54.09–107.98)		
Last sample <sup>§</sup>		53.81 (34.87–79.83)	75.37 (59.65–118.00)	47.59 (32.98–64.18)	<0.001*
Baseline sample	NT-proBNP	8.90 (3.82–16.93)	18.48 (11.19–33.71)	6.32 (2.82–12.39)	<0.001*
Second-last sample <sup>‡</sup>			21.28 (12.75–45.30)		
Last sample <sup>§</sup>		8.54 (3.24–19.31)	26.69 (12.61–53.77)	5.68 (2.66–12.82)	<0.001*

Biomarker candidates are presented as median (25th–75th percentile). AP-N indicates aminopeptidase-N; CASP3, caspase-3; CSTB, cystatin-B; CTSD, cathepsin D; CTZ, 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.

\*P value below the corrected significance level for multiple testing ( $P<0.005$ ).

<sup>†</sup>Median concentrations in this table are given on the linear scale in arbitrary (relative) units. Although the assay used does not provide absolute levels, the relative levels do enable us to investigate whether a rise or fall in candidate biomarker level (and slope) is associated with adverse clinical outcome.

<sup>‡</sup>Second-last sample before the primary end point.

<sup>§</sup>Last sample indicates the last sample before the primary end point or last sample available for patients who did not reach the primary end point.

of the end point and is depicted on the right side of the x-axis; inherently to this representation, baseline sampling preceded this “time zero”). Furthermore, ST2 significantly increased as the end point approached, but remained stable in end point–free patients. All biomarker candidates, except for caspase-3 and cathepsin Z, showed a similar pattern although sometimes less obvious.

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), which implies that if a patient has a 1 SD higher ST2 level compared with another patient at any point in time, the HR for that patient of a PE is 7.55. ST2 was 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, perlecan, AP-N, CTSD, cystatin-B, and NT-proBNP independently predicted the end point (all  $P<0.005$ ). Furthermore, levels of these biomarker candidates, except for MMP-3 and AP-N, remained significant predictors after adjusting for established cardiac markers NT-proBNP and hsTnT, where ST2 (HR 4.02, 95% CI 2.56–7.07), GDF-15 (HR 2.50, 95% CI 1.83–3.48), and MMP-2 (HR 2.45, 95% CI 1.66–3.75) showed the strongest associations with the PE.

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, perlecan, 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) ( $P<0.005$ , for HRs see Table 4). After adjusting for clinical characteristics and HF medication (clinical and time-varying medication model), the slope of MMP-2 was the numerically strongest predictor of the PE (HR 1.18 per 0.1 SD/y difference, 95% CI 1.10–1.26). The slope of TIMP-4 showed the strongest association with the PE (HR 1.23, 95% CI 1.15–1.34) after adjusting for established cardiac biomarkers (NT-proBNP and hsTnT).

## 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, perlecan, CTSD, and CSTB) increase markedly and significantly as an adverse clinical event approaches. Several biomarkers, including ST-2, Gal-4, GDF-15, perlecan, and CSTB already show differences at baseline between patients who will reach the primary end point versus those who will remain event free. Importantly, however, the repeatedly measured levels of biomarker candidates of cardiac remodeling predict incident adverse clinical events, with ST2, GDF-15, and MMP-2 being the strongest predictors. Independently of their levels, the rate of biomarker change over time of the biomarker candidates also predicts incident events, with MMP-2, MMP-9, and TIMP-4 being strong predictors. The clinical implications of this slope are particularly important in situations where, for instance, at a specific time point 2 patients show similar marker levels, but have different rates of change of the marker. Above-described associations persist after multivariable adjustment for clinical characteristics, pharmacological treatment during

**Table 3.** Associations Between Blood Biomarkers of Cardiac Remodeling and the Primary End Point: Levels

	Crude Model		Clinical Model		Clinical and Time-Varying Medication Model		Established 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*

Hazard ratios (HRs) and 95% CIs are given per SD increase at any point in time during follow-up, which were estimated by joint modeling (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. This statistical approach enabled us to simultaneously take into account all individual values of the available measurements in the current analyses (ie, all baseline samples, the last sample available in patients in whom the primary end point [PE] did not occur during follow-up, and the 2 samples available closest in time before the PE). Crude model: Cox model unadjusted, LME model adjusted for sampling time; Clinical model: Cox and LME models adjusted for age, sex, diabetes mellitus, atrial fibrillation, baseline New York Heart Association 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 of equivalents of carvedilol, enalapril, furosemide, and spironolactone during follow-up; Established cardiac biomarker model: Cox and LME models adjusted for baseline NT-proBNP and hsTnT, and sampling time (LME). Data on all variables were complete, except for systolic blood pressure, which was missing in >5% of patients and for which imputations were applied using the patients' clinical and outcome data. x not performed because repeatedly measured level was not significant. AP-N indicates aminopeptidase-N; CASP3, caspase-3; CSTB, cystatin-B; CTSD, cathepsin D; CTSZ, cathepsin Z; eGFR, estimated glomerular filtration rate; Gal-3, galectin-3; Gal-4, galectin-4; GDF-15, growth differentiation factor 15; HR, hazard ratio; hsTnT, highly sensitive cardiac troponin T; MMP-2, 3, and 9, matrix metalloproteinase 2, 3, and 9; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PLC, perlecan; ST2, suppression of tumorigenicity-2; TIMP-4, tissue inhibitor metalloproteinase 4.

\*P value below the corrected significance level for multiple testing ( $P < 0.005$ ).

follow-up, and cardiac biomarkers NT-proBNP and hsTnT, indicating that these markers carry incremental value for prediction of future CHF events in comparison to the established (bio)markers that are currently used in clinical practice.

ST2 is an interleukin-1 receptor family member whose ligand is the cardioprotective interleukin-33. An increase of the soluble circulating form of ST2 binds interleukin-33 and makes it unavailable to the ST2 receptor for cardioprotective signaling. This results in increased myocardial apoptosis, fibrosis, and hypertrophy.<sup>31</sup> Higher ST2 plasma concentrations have been 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.<sup>31</sup> Accordingly, the updated guidelines for the management of HF suggest the use of ST2 for risk stratification in CHF patients.<sup>32</sup> 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,<sup>10–12</sup> but certain limitations restricted their generalizability. One study had a relatively short follow-up period of 10 months after recent HF decompensation<sup>11</sup>; 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.<sup>10,12</sup> 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.<sup>9</sup> 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

**Table 4.** Associations Between Blood Biomarkers of Cardiac Remodeling and the Primary End Point: Slopes

	Crude Model		Clinical Model		Clinical and Time-Varying Medication Model		Established 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/y 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% CIs are given per 0.1 SD of the annual slope at any point in time during follow-up, which were estimated by joint modeling (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. This statistical approach enabled us to simultaneously take into account all individual values of the available measurements in the current analyses (ie, all baseline samples, the last sample available in patients in whom the primary end point [PE] did not occur during follow-up, and the 2 samples available closest in time before the PE). Annual slopes were additionally adjusted for the levels of repeatedly measured marker 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 mellitus, atrial fibrillation, baseline New York Heart Association 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 of equivalents of carvedilol, enalapril, furosemide, and spironolactone during follow-up; Established cardiac biomarker model: Cox and LME models adjusted for baseline NT-proBNP and hsTnT, and sampling time (LME). Data on all variables were complete, except for systolic blood pressure, which was missing in >5% of patients and for which imputations were applied using the patients' clinical and outcome data. x not performed because repeatedly measured level was not significant. AP-N indicates aminopeptidase-N; CASP3, caspase-3; CSTB, cystatin-B; CTSD, cathepsin D; CTSZ, cathepsin Z; eGFR, estimated glomerular filtration rate; Gal-3, galectin-3; Gal-4, galectin-4; GDF-15, growth differentiation factor 15; hsTnT, highly sensitive cardiac troponin T; MMP-2, 3, and 9, matrix metalloproteinase 2, 3, and 9; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PLC, perlecan; ST2, suppression of tumorigenicity-2; TIMP-4, tissue inhibitor metalloproteinase 4.

\*P value below the corrected significance level for multiple testing ( $P<0.005$ ).

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 2 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 prespecified study protocol at fixed 3-month intervals over the full course of follow-up, with up to 11 samples per patient. This enabled us to select the 2 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 emphasizes the incremental value of serial ST2 measurements.

Gal-3 is a soluble  $\beta$ -galactoside-binding lectin and a member of the galectin family<sup>33</sup> and this biomarker is deemed a relevant mediator in the cardiac remodeling process.<sup>34</sup> A recent meta-analysis showed that increased Gal-3 levels carry higher risk of mortality independently of well-established risk factors.<sup>35</sup> Nevertheless, whether this association between Gal-3 and adverse outcome is independent of natriuretic peptides remained unclear.<sup>36–38</sup> 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 (Controlled Rosuvastatin Multinational Trial in Heart Failure) trial (baseline measurement plus additional measurement after 3 months) and the COACH (Coordinating Study Evaluating Outcomes of Advising and Counseling Failure) trial (baseline measurement plus additional measurement after 6 months).<sup>9</sup> 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- $\beta$  superfamily, may influence cardiac remodeling via 2 different mechanisms (ie, protection from apoptosis and induction of hypertrophy).<sup>39</sup> Several studies have shown promising results on the prognostic value of GDF-15. Chan et al<sup>40</sup> found prognostic utility of GDF-15 measured at 6 weeks and 5 months beyond NT-proBNP in both HF patients with reduced ejection fraction and those with preserved ejection fraction. In the HF-ACTION Study (HF patients with reduced ejection fraction),<sup>7</sup> 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 perlecan and AP-N. Several MMPs and TIMPs are associated with fibrosis, diastolic dysfunction, and left ventricular hypertrophy,<sup>41,42</sup> and some of these, such as MMP-9 and TIMP-1, correlated with the severity of CHF.<sup>43</sup> Moreover, MMPs are implicated in several cardiovascular diseases; for example, MMP-2 and -9 are potential biomarkers of acute myocardial infarction<sup>44</sup> and coronary artery disease.<sup>45</sup> 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.<sup>46</sup> In our study MMP-2, MMP-9, TIMP-4, and perlecan were clear predictors of the PE. Conversely, level and slope of MMP-3 were not significant predictors of adverse events after adjustment. AP-N is a type II metalloprotease,<sup>47</sup> 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 (ie, 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 are available on biomarkers of apoptosis, such as caspase-3, CTSD, cathepsin Z, 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% were in NYHA class I-II. Still, we were able to show that biomarker candidates of cardiac remodeling are 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.

Our study has several limitations. First, as described before,<sup>16</sup> our cohort comprised mainly HF patients with reduced ejection fraction. This can most likely be attributed to the fact that in the Netherlands, most HF patients with preserved ejection fraction are followed in secondary referral centers or by the general practitioner, while the current study was performed in 2 tertiary referral centers. Second, 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,<sup>15</sup> we have used all available sampling moments to determine NT-proBNP, hsTnT, C-reactive protein as well as glomerular and tubular renal biomarkers.<sup>16</sup> Those investigations demonstrated that most of these biomarker candidates show an increase shortly before the incident adverse event. Thus, we believe that by selecting baseline samples, as well as the last 2 samples before the incident end point, we retain the most informative measurements while enhancing efficiency. Finally, the assay we used for measuring the biomarker candidates 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.

In conclusion, this study shows that temporal patterns of patient-specific levels of numerous biomarker candidates of cardiac remodeling predict clinical outcome in CHF; specifically, these remodeling biomarker candidates increase before an adverse event in CHF patients. These patient-specific temporal patterns, in particular of levels of ST-2, Gal-3, and GDF-15, and of rate of change in MMP-2, MMP-9, and TIMP-4,



indicate a promising role of these biomarker candidates for individual prognostication and treatment monitoring.

## Sources of Funding

This work was supported by the Jaap Schouten Foundation and the Noordwest Academie.

## Disclosures

Mouthaan is employed by Olink Proteomics. The remaining authors have no disclosures to report.

## References

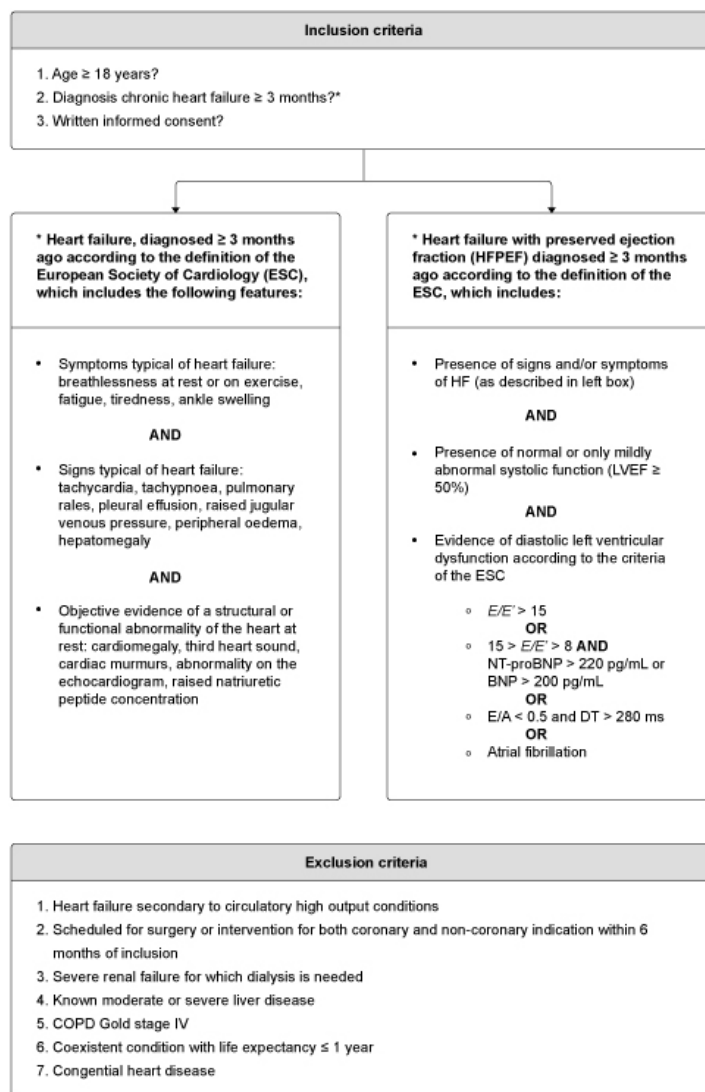
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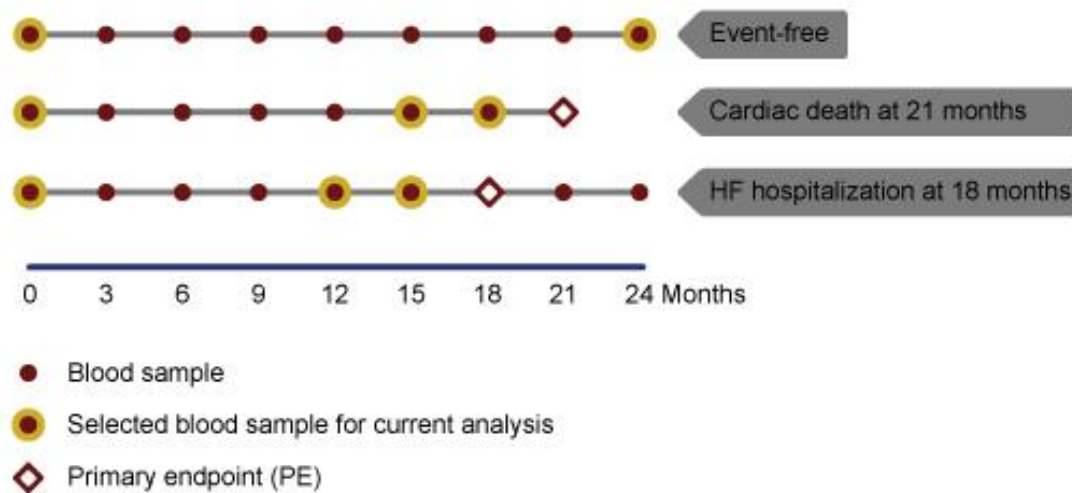
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# **SUPPLEMENTAL MATERIAL**

**Figure S1. Inclusion and exclusion criteria.**

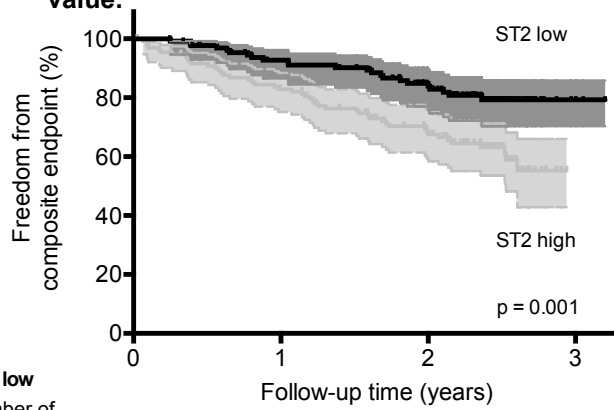


**Figure S2. Selection of blood samples.**

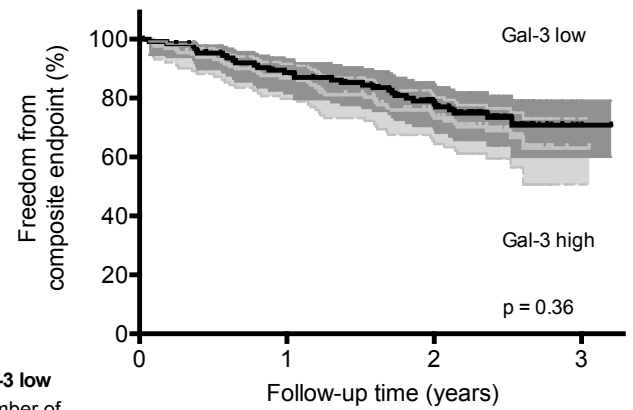


At each study follow-up visit, the research physician performed a short medical evaluation and blood samples were collected. Study follow-up visits were predefined and scheduled every 3 months ( $\pm 1$  month). The primary endpoint (PE) was a composite of cardiac death, heart transplantation, left ventricular 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. As depicted in this Figure S2, 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.

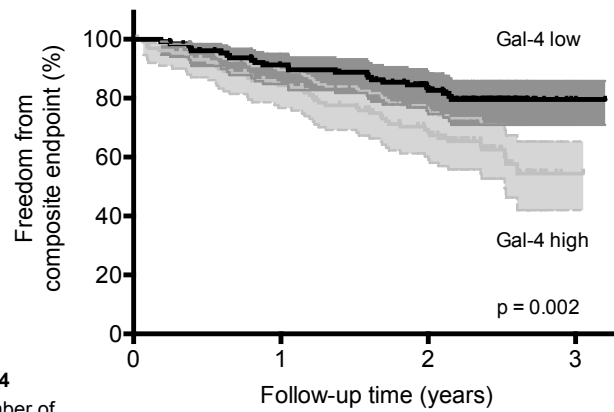
**Figure S3. Freedom from composite endpoint for all biomarker-candidates above and below median value.**



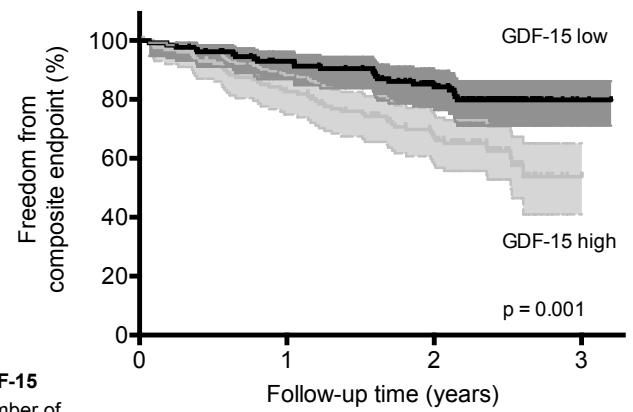
<b>ST2 low</b>				
Number of remaining patients:	130	111	85	54
<b>ST2 high</b>				
Number of remaining patients:	131	105	74	15



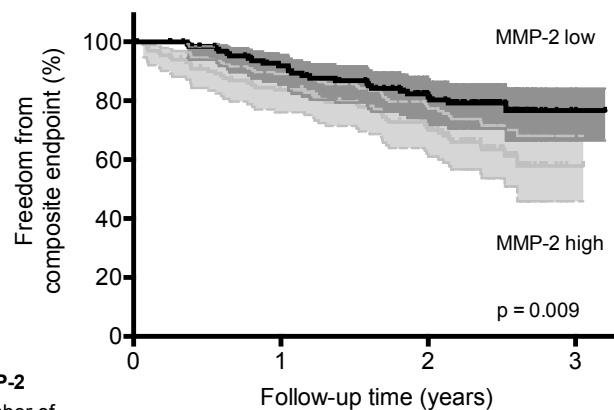
<b>Gal-3 low</b>				
Number of remaining patients:	130	105	79	27
<b>Gal-3 high</b>				
Number of remaining patients:	131	109	80	17



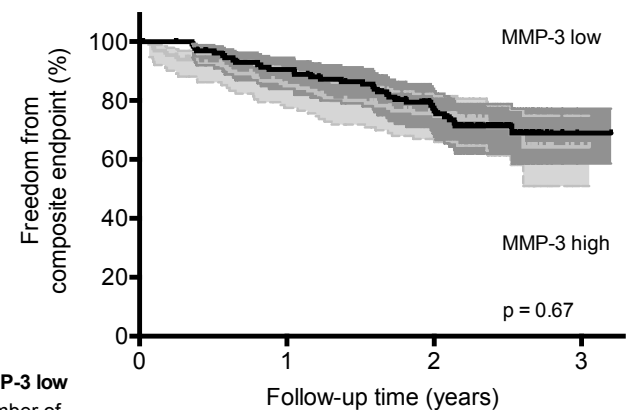
<b>Gal-4</b>				
Number of remaining patients:	131	112	88	75
<b>Gal-4</b>				
Number of remaining patients:	130	103	71	17



<b>GDF-15</b>				
Number of remaining patients:	131	114	86	72
<b>GDF-15</b>				
Number of remaining patients:	130	102	73	14

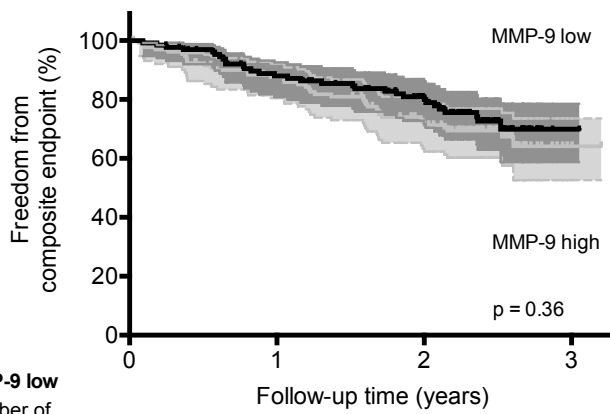


<b>MMP-2</b>				
Number of remaining patients:	131	112	80	30
<b>MMP-2</b>				
Number of remaining patients:	130	105	79	17



<b>MMP-3 low</b>				
Number of remaining patients:	131	112	78	29
<b>MMP-3 high</b>				
Number of remaining patients:	130	103	83	15





#### MMP-9 low

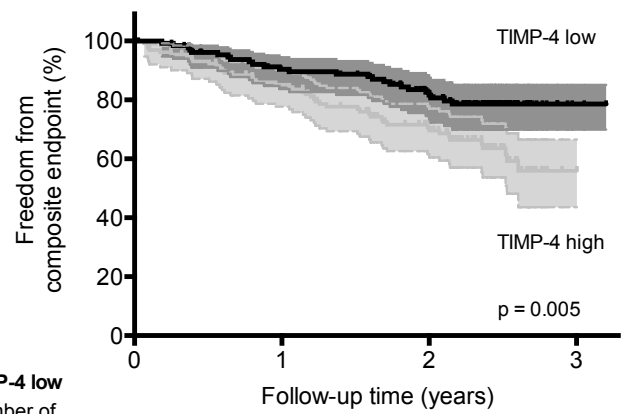
Number of remaining patients:

130      106      80      25

#### MMP-9 high

Number of remaining patients:

131      108      79      20



#### TIMP-4 low

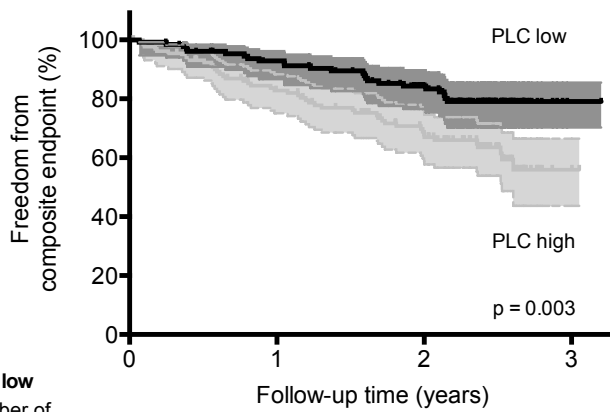
Number of remaining patients:

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#### TIMP-4 high

Number of remaining patients:

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#### PLC low

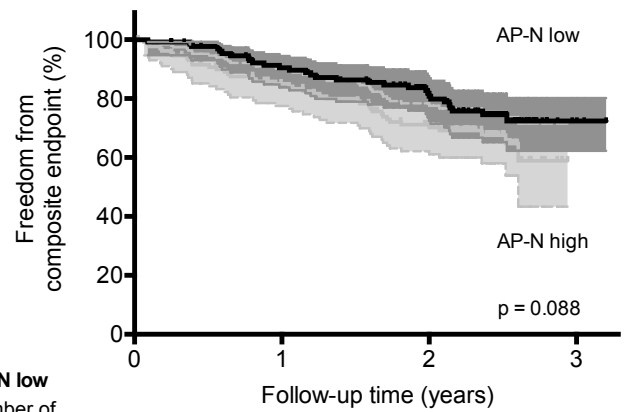
Number of remaining patients:

130      114      95      73

#### PLC high

Number of remaining patients:

131      102      72      16



#### AP-N low

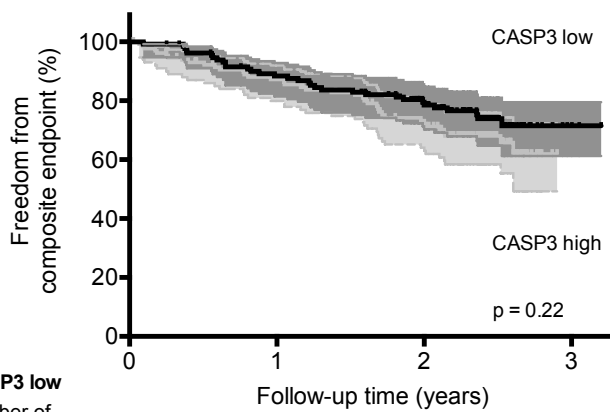
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131      111      83      34

#### AP-N high

Number of remaining patients:

130      103      78      10



#### CASP3 low

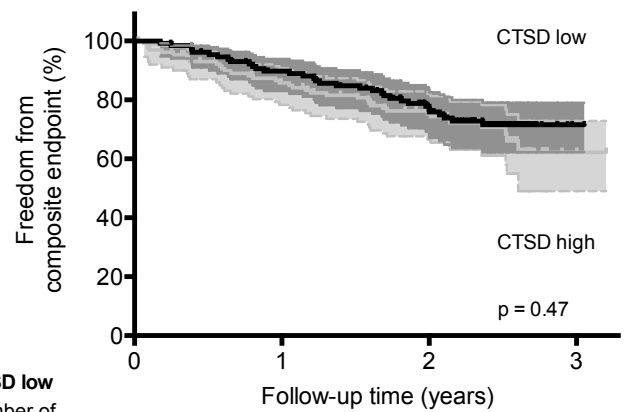
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131      112      85      30

#### CASP3 high

Number of remaining patients:

130      102      74      16



#### CTSD low

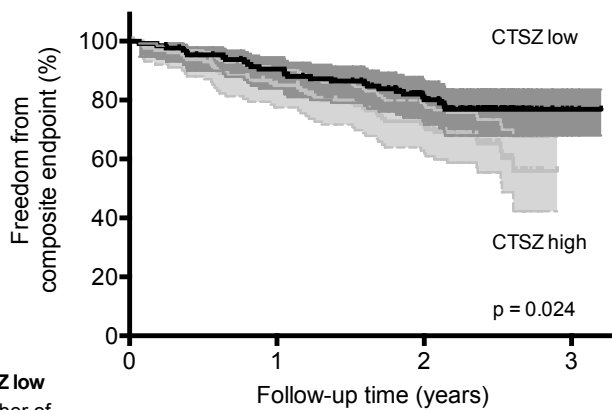
Number of remaining patients:

131      111      79      58

#### CTSD high

Number of remaining patients:

130      105      80      16



#### CTSZ low

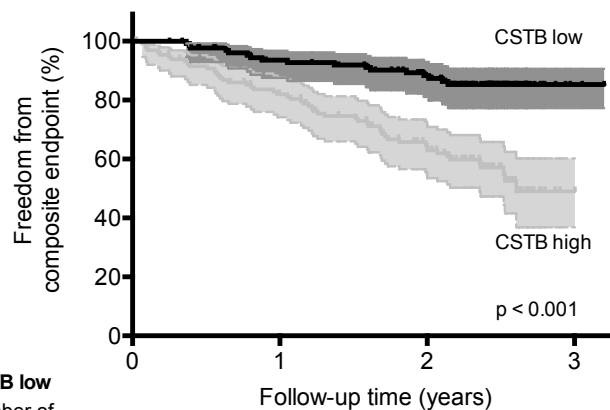
Number of remaining patients:

131      112      81      71

#### CTSZ high

Number of remaining patients:

130      103      78      13



#### CSTB low

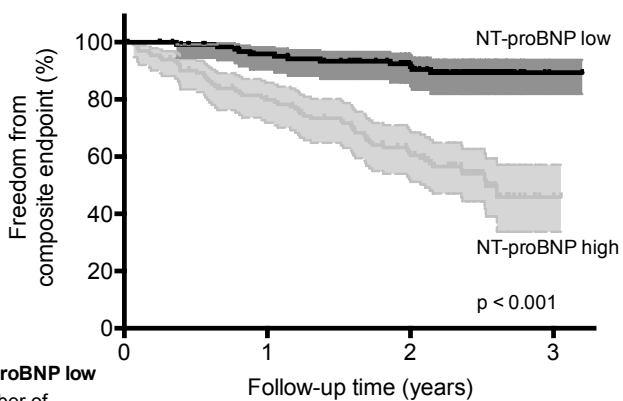
Number of remaining patients:

131      114      94      80

#### CSTB high

Number of remaining patients:

130      102      67      14



#### NT-proBNP low

Number of remaining patients:

131      115      92      82

#### NT-proBNP high

Number of remaining patients:

130      101      67      12