ESC HEART FAILURE
ESC Heart Failure 2020; **7**: 1190–1200
Published online 20 March 2020 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/ehf2.12678

ORIGINAL RESEARCH ARTICLE

Temporal patterns of macrophage- and neutrophil-related markers are associated with clinical outcome in heart failure patients

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Abstract

Aims Evidence on the association of macrophage- and neutrophil-related blood biomarkers with clinical outcome in heart failure patients is limited, and, with the exception of C-reactive protein, no data exist on their temporal evolution. We aimed to investigate whether temporal patterns of these biomarkers are related to clinical outcome in patients with stable chronic heart failure (CHF).

Methods and Results In 263 patients with CHF, we performed serial plasma measurements of scavenger receptor cysteine-rich type 1 protein M130 (CD163), tartrate-resistant acid phosphatase type 5 (TRAP), granulins (GRN), spondin-1 (SPON1), peptidoglycan recognition protein 1 (PGLYRP1), and tissue factor pathway inhibitor (TFPI). The Cardiovascular Panel III (Olink Proteomics AB, Uppsala, Sweden) was used. During 2.2 years of follow-up, we collected 1984 samples before the occurrence of the composite primary endpoint (PE) or censoring. For efficiency, we selected 567 samples for the measurements (all baseline samples, the last two samples preceding the PE, and the last sample before censoring in event-free patients). The relationship between repeatedly measured biomarker levels and the PE was evaluated by joint models. Mean (±standard deviation) age was 67 ± 13 years; 189 (72%) were men; left ventricular ejection fraction (%) was 32 ± 11. During follow-up, 70 (27%) patients experienced the PE. Serially measured biomarkers predicted the PE in a multivariable model adjusted for baseline clinical characteristics [hazard ratio (95% confidence interval) per 1-standard deviation change in biomarker]: CD163 [2.07(1.47–2.98), $P < 0.001$], TRAP [0.62 (0.43–0.90), $P = 0.009$], GRN [2.46 (1.64–3.84), $P < 0.001$], SPON1 [3.94 (2.50–6.50), $P < 0.001$], and PGLYRP1 [1.62 (1.14–2.31), $P = 0.006$].

Conclusions Changes in plasma levels of CD163, TRAP, GRN, SPON1, and PGLYRP1 precede adverse cardiovascular events in patients with CHF.

Keywords Scavenger receptor cysteine-rich type 1 protein M130; Tartrate-resistant acid phosphatase type 5; Granulins; Spondin-1; Peptidoglycan recognition protein

Received: 16 November 2019; Revised: 17 February 2020; Accepted: 22 February 2020
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Introduction

The role of the immune system in heart failure is gaining attention.[1] In general, the innate immune system provides a non-specific defence against pathogens or tissue injury. It comprises various soluble molecules (e.g. complement system or pentraxins) and multiple cellular components, including antigen presenting cells (e.g. dendritic cells), phagocytic cells (e.g.

neutrophils and macrophages), and natural killer cells.[2] These immune cells express germ-line encoded pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns [1] and either activate the NFkB pathway to increase the expression of cytokines and interferons, or trigger formation of inflammasome complexes which subsequently mediate the production of interleukin-1B and interleukin-18.[3]

In the context of heart failure, several types of PRRs have been implicated in myocardial remodelling, including plasma- and endosome-bound toll-like receptors type 4, as well as cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors NOD containing protein 1 and NOD-, LRR- and pyrin domain-containing protein 3.[3,4] Additionally, activation of the immune system may occur in extra-cardiac tissues as a result of failure of the heart to maintain sufficient blood flow and subsequent tissue damage. This systemic inflammation may enhance unfavourable myocardial remodelling as well.[5] For several heart failure-attributed biomarkers (such as inflammatory-linked galectin-3, growth differentiation factor-15, and tissue inhibitor of metalloproteinase-1), extra-cardiac origins have been demonstrated.[6]

However, PRRs are only a part of the whole abundance of molecules involved in the action of innate immune system, which could play a role in myocardial remodelling and failure. With this study, our main aim was to investigate the relationship between clinical outcomes of heart failure patients and serially measured plasma levels of molecules involved in the actions of the innate immune system. In our previous investigations, we have examined other components of the proteomic multiplex panel by categorizing the 92 biomarkers based on their role in various processes (cardiometabolic regulation, myocardial remodelling and extracellular matrix turnover, fibrinolysis, and renal and pulmonary function. The current analysis specifically includes biomarkers related to the action of two cellular players of the innate immune system: macrophages and, to a lesser extent, neutrophils. We describe cytokines and chemokines elsewhere; moreover, we assigned those molecules that, besides their function in the innate immunity, play important roles in other processes, to the pathways listed above; such as for example galectin-3 or suppression of tumorigenicity 2 .[7–9] Here, we aim for a deeper understanding of innate immune system dynamics in patients with heart failure and to investigate the utility of macrophage- and neutrophil-related molecules as biomarkers of prognosis in these patients. Specifically, we examined the following molecules that were available on a multiplex assay: peptidoglycan recognition protein 1 (PGLYRP1; actions of neutrophils), scavenger receptor cysteine-rich type 1 protein M130 (CD163), tartrate-resistant acid phosphatase type 5 (TRAP), granulins (GRN), and spondin-1 (SPON1) (macrophages) as well as tissue factor pathway inhibitor (TFPI) (tissue response to aforementioned cells).

Methods

Chronic heart failure cohort

The Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis (Bio-SHiFT) is a prospective cohort study of stable patients with chronic heart failure (CHF) conducted in Erasmus MC, Rotterdam, and Northwest Clinics, Alkmaar, Netherlands. Ambulatory adult patients were included if chronic heart failure had been diagnosed according to European Society Guidelines at least 3 months before and the clinical course was stable [i.e. they had not been hospitalized for heart failure (HF) in the past 3 months]. Exact inclusion and exclusion criteria are presented in Supporting Information *Figure S1*. The study was approved by the medical ethics committees, conducted in accordance with the Declaration of Helsinki,[10] and registered in ClinicalTrials.gov (NCT01851538). All the included patients signed informed consent. Analyses presented in this article comprised 263 patients with CHF enrolled during the first inclusion round period (October 2011 until June 2013).

Baseline assessment and follow-up procedures

All patients were evaluated by research physicians, who collected information on HF-related symptoms and medical history, and performed a physical examination. Patients were categorized using National Kidney Foundation–Kidney Disease Outcome Quality Initiative clinical practice guidelines on the basis of glomerular filtration rate (GFR) estimated with the chronic kidney disease epidemiology collaboration equation.

Study follow-up visits were predefined and scheduled every 3 months (±1 month). Routine visits at the outpatient clinic continued in parallel and were performed by the treating physicians, who were blinded for biomarker results. 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 discharge letters were collected. Subsequently, a clinical event committee, blinded to the biomarker results, reviewed hospital records and discharge letters and adjudicated the study endpoints.

The primary endpoint (PE) was a composite of cardiac death, heart transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened HF, whichever occurred first. Additionally, the secondary endpoint consisting of cardiac death and

hospitalization for the management of acute or worsened HF was evaluated. We used the WHO International Classification of Disease-10th revision, to assign the fatal endpoints. Cardiac death was defined as death from myocardial infarction or other ischemic heart disease (International Classification of Disease-10th revision: 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 (Northwest Clinics) or N-terminal pro BNP (NT-proBNP) (Erasmus MC) >3× upper limit of normal (105 ng/L and 45 pmol/l, respectively), signs of worsening HF, such as pulmonary rales, raised jugular venous pressure, or peripheral oedema, increased dose or intravenous administration of diuretics, or administration of positive inotropic agents.[11]

Blood sampling

Blood samples were collected at baseline and at each trimonthly study follow-up visit. They were processed and stored at −80°C within 2 h after collection until batchwise biomarker measurement was performed after completion of the follow-up. 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 in 263 patients (median [25th–75th percentile] 9[5–10] blood samples per patient). Our previous analyses using all available samples in this cohort have demonstrated that the concentration of several plasma and urine biomarkers changes over the months preceding the incident adverse event. For reasons of efficiency, for the current investigation, 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. We assumed that by selecting the last two samples prior to the incident endpoint, we would capture the change in biomarkers in patients with an event. Conversely, in event-free patients, our previous investigations showed stable biomarker levels; in which case, one additional sample suffices.[12] Altogether, our selection amounted to 567 samples for the current analysis. All laboratory personnel were blinded for clinical data and patient outcomes.

Laboratory measurements

The cardiovascular (cardiovascular disease) 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 one plasma sample. The proteins analysed 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.[13] The biomarkers are delivered in normalized protein expression (NPX) units, which are relative units that result from the real-time polymerase chain reaction. They are expressed on a log2 scale so that a 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 for comparing fold changes between groups. In the current analysis, CD163, TRAP, GRN, SPON1, PGLYRP1, and TFPI were investigated, because these proteins are involved in innate immune system functioning. Measurements below the limit of detection were only observed in case of SPON1 (n = 38, 6.6% of all measurements).

Plasma NT-proBNP was analysed using an electrochemiluminescence immunoassay (lower limit of detection, LLD 5 ng/L, Elecsys 2010; Roche Diagnostics, Indianapolis, IN). High-sensitivity cardiac troponin T was also measured using an electrochemiluminescence immunoassay (LLD 3 ng/L, Elecsys 2010 immunoassay analyser; Roche Diagnostics, Indianapolis, IN). C-reactive protein (CRP) was analysed using an immunoturbidimetric assay (LLD 0.3 mg/L, Roche Hitachi 912 chemistry analyser; Roche, Basel, Switzerland). Creatinine was determined by a colorimetric test by the Jaffe's reaction in undiluted plasma (LLD: 0,14 mg/dL). All coefficients of variation of these four biomarkers were below 5%.

Statistical analysis

Variables with a normal distribution are presented as mean ± standard deviation (SD), whereas the median and interquartile range (25th–75th percentile) are presented in case of non-normality. Categorical variables are presented as counts and percentages. For the analysis, in order to allow for direct comparisons between different biomarkers we used the Z-score (i.e. the mean was subtracted from the raw value, subsequently divided by the SD) of the biomarker levels delivered in NPX units.

We evaluated the associations between baseline biomarker levels and the PE with the use of univariable and multivariable Cox proportional hazard regression models. We adjusted the biomarker-outcome relation for age, sex, diabetes mellitus (DM), atrial fibrillation (AF), baseline New York Heart Association (NYHA) class, systolic blood pressure (SBP), estimated GFR, and CRP. Then, we evaluated the associations between repeated biomarker measurements and the PE with joint models (JMs) thus combining the linear mixed effects (LME) models for repeated measurements with relative risk models for time-to-event data, including covariates as described

above.[14] Finally, we used time-dependent Cox models adjusted for the same covariates as in the JM and additionally adjusted for changing total daily equivalent doses of HF medication used during follow-up (carvedilol, enalapril, spironolactone, and furosemide), as defined in *Table S2*. Results are given as hazard ratios and 95% confidence intervals per 1 SD difference of the biomarker level expressed in NPX units. To plot the average temporal patterns of immune biomarkers in patients with and without the PE, we also used LME models. We applied the Bonferroni correction method (threshold for *P* value: 0.008) to correct for multiple testing.

Additionally, we studied the associations of baseline characteristics and routinely used biomarkers (NT-proBNP, high-sensitivity troponin T, and CRP) with repeatedly measured immune biomarkers with the use of a LME model. Immune biomarkers were entered as the dependent variables and sampling time (fixed and random effect), baseline characteristics (fixed effects), and routine biomarkers (fixed effects) as the independent variables. All the investigated baseline characteristics were concomitantly entered into one multivariable model for each biomarker, so that any observed associations would be independent of the other characteristics. Here, the conventional threshold was used for the *P* value (<0.05).

Data on all variables used in the models were complete, except for SBP, which was missing in $<5\%$ of patients and for which imputations were applied using the patients' clinical and outcome data. Adjusted and unadjusted models were nested.

We have used the Panther Database to verify the molecular function and involvement in biological processes of the biomarkers that we investigated.[15]

All tests were two-tailed. All analyses were performed with R statistical software v. 3.4.1. using packages nlme v. 3.1-137 and JMbayes v. 0.8-71.

Results

Baseline characteristics and clinical outcomes

Mean age was 67 ± 13 years; 189 (72%) were men, 69 (26%) in NYHA class III or IV, and mean left ventricular ejection fraction (LVEF) was 32 ± 11%. *Table 1* presents baseline characteristics of the study population. Among baseline characteristics analysed univariably with a Cox model, the following were associated significantly with the PE: age, NYHA class, DM, SBP, NT-proBNP, high-sensitivity troponin T, CRP and diuretic dose, as presented in *Table S1*.

Follow-up and study endpoints

During a median (interquartile range) follow-up of 2.2 (1.4–2.5) years, a total of 70 (27%) patients reached the composite PE: 56 of these 70 endpoints (80%) consisted of rehospitalization for acute or worsened HF, 3 (4%) consisted of heart transplantation, 2 (3%) of left ventricular assist device placement, and 9 (13%) of cardiovascular mortality. The secondary endpoint was observed in 65 patients.

Temporal evolution of immune biomarkers in relation to study endpoints

The average temporal trajectories of immune biomarkers in patients who experienced an incident endpoint and those who did not are displayed in *Figure 1*. CD163, GRN, SPON1, and PGLYRP1 increased prior to the occurrence of an endpoint, whereas TRAP decreased and TFPI level remained almost constant. At the same time, the biomarker levels remained relatively stable or showed smaller change in event-free patients.

Table 2 presents the associations of the immune biomarkers with the PE. After adjustment for clinical covariates, the occurrence of the PE was independently predicted by repeated measurements of CD163 (hazard ratio [95% confidence interval] per 1 SD change of the biomarker level: 2.07 [1.47–2.98], $P < 0.001$, TRAP 0.60 [0.45–0.80], $P < 0.001$, GRN 2.46 [1.64–3.84], $P < 0.001$, SPON1 (3.94 [2.50–6.50], $P < 0.001$, and PGLYRP1 (1.62 [1.14–2.31], $P = 0.006$). These associations persisted also after adjustment for changes in medication dosage over follow-up and were valid also if only patients with reduced ejection fraction (HF with reduced ejection fraction) were analysed (effect estimates are displayed in *Table S5*). The same set of repeatedly measured biomarkers independently predicted the occurrence of the secondary endpoint in both clinically- and medication-adjusted models (*Table S6*). As the major etiologic factor in our study population was ischemic heart disease, the association between the investigated biomarkers and the PE was evaluated separately in this subgroup, with CD163, TRAP, SPON1, and PGLYRP1 showing independent predictive value (*Table S7*). For the second-largest etiologic subgroup, dilated cardiomyopathy, no associations could be demonstrated between baseline biomarker levels and the PE, and most of the JMs did not converge properly probably because the size of the subgroup was too small and the number of events too low. Therefore, some estimates could not be calculated, and those that are presented should be interpreted with reservation (*Table S8*).

Association between immune biomarkers and baseline characteristics

Some of the baseline characteristics were independently associated with longitudinal patterns of the investigated biomarkers (*Table 3*). Older age was associated with higher

Table 1 Baseline characteristics of the study population ($N = 263$)

Variable	Value
Demographics	
Age (years)	67 ± 13
Male gender	189 (72)
Clinical characteristics	
BMI (kg/m2)	27.5 ± 4.7
Systolic blood pressure (mmHg)	122 ± 20
Diastolic blood pressure (mmHg)	72 ± 11
Pulse (beat/min)	67 ± 12
Features of HF	
LVEF, %	32 ± 11
NYHA class III or IV	69 (26)
Aetiology of HF	
Ischemic heart disease	117 (44)
Hypertension	34 (13)
Secondary to valvular disease	12 (5)
Cardiomyopathy	68 (26)
Other or unknown	32 (12)
Medical history	
Myocardial infarction	96 (37)
PCI	82 (31)
CABG	43 (16)
AF	106 (40)
Hypertension	120 (46)
Diabetes mellitus	81 (31)
Known hypercholesterolemia	96 (37)
COPD	31 (12)
KDOQI classification	
eGFR ≥90 mL/min/1.73 m2	28 (11)
eGFR 60–89 mL/min/1.73 m2	95 (36)
eGFR 30–59 mL/min/1.73 m2	119 (45)
eGFR <30 mL/min/1.73 m2	21 (8)
Medication use	
Beta-blocker	236 (90)
ACE-I or ARB	245 (93)
Aldosterone antagonist	179 (68)
Diuretic	237 (90)
Loop diuretics	236 (90)
Thiazides	7 (3)
Biomarker concentrations	
Cardiac	
NT-proBNP (pmol/L)	137.3 (51.7–272.6)
Hs-TnT (ng/L)	18.0 (9.5–33.2)
Inflammatory	
CRP (mg/L)	2.2 (0.9–4.8)
Glomerular	
Creatinine, mg/dl	1.18 (0.99–1.49)
eGFR-CKD EPI, mL/min per 1.73 m2	58 (43–76)
Immune response biomarkers[a]	
CD163	6.24 (5.92–6.62)
TRAP	4.39 (4.16–4.65)
GRN	6.08 ± 0.41
SPON1	0.83 (0.66–1.13)
TFPI	8.23 (7.92–8.57)
PGLYRP1	7.17 ± 0.63

ACE-I, angiotensin-converting enzyme inhibitor; AF, atrial fibrillation; ARB, angiotensin II receptor blocker; BMI, body mass index; CABG, coronary artery bypass grafting; CD163, scavenger receptor cysteine-rich type 1 protein M130; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CRP, C-reactive protein; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GRN, granulins; Hs-TnT, high-sensitivity troponin T; KDOQI, Kidney Disease Outcomes Quality Initiative; LVEF, left ventricular ejection fraction; MI, myocardial infarction; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; PCI, percutaneous coronary intervention; PGLYRP1, Peptidoglycan recognition protein 1 (TAG7); SBP, systolic blood pressure. SPON1, spondin-1; TFPI, tissue factor pathway inhibitor; TRAP, tartrate-resistant acid phosphatase type 5.

Categorical variables are expressed as count (percentage). Values of continuous variables are expressed as mean ± standard deviation or as median (interquartile range) in case of skewed distribution.

[a]Immune biomarker concentrations in this table are given in arbitrary normalized protein expression (relative) units on a log2 scale.

levels of TRAP and lower levels of in TFPI during follow-up. Patients with DM and AF had higher CD163, GRN, and SPON1 during follow-up. Lower estimated GFR was associated with higher GRN, SPON1, TFPI, and PGLYRP1. *Table 3* also summarizes the association between routinely used biomarkers and immune biomarkers: a positive association was present between CRP and CD163, GRN, and SPON1; NT-proBNP showed positive associations with GRN, SPON1, and PGLYRP1 and inverse associations with TRAP and TFPI, whereas higher troponin was associated only with higher PGLYRP1. Higher baseline diuretics doses were associated positively with plasma SPON1, whereas higher spironolactone dose was associated with lower levels of CD163, GRN, SPON1, TFPI, and PGLYRP1.

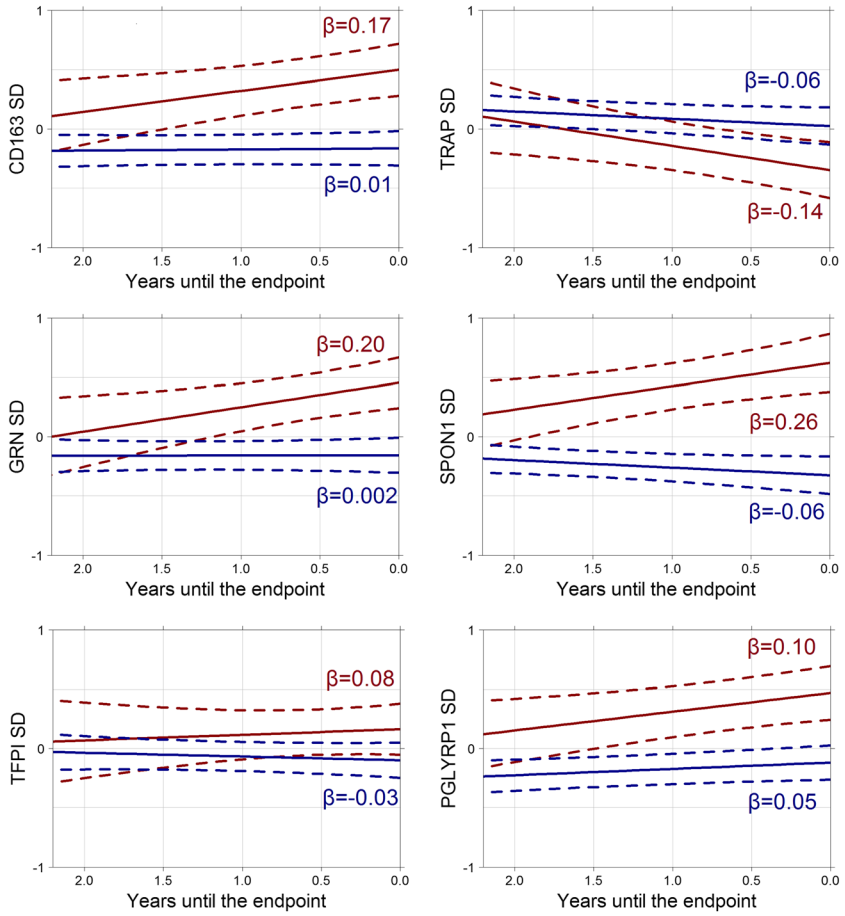
Baseline values of immune biomarkers showed a moderate degree of correlation in most cases, which can be observed in *Figure 2*. Coefficient values are presented in *Table S3*. The molecular function of the biomarkers and their involvement in biological processes as derived from the Panther Database is summarized in *Table S4*.

Discussion

In this prospective, observational study, we demonstrate that the temporal evolutions of the investigated macrophage and neutrophil-related biomarkers CD163, TRAP, GRN, SPON-1, and PGLYRP-1 show significant and independent associations with adverse clinical outcomes in CHF patients. Additionally, we observe that these biomarkers reflect the severity of heart failure as all of them, except for CD163, correlate with NT-proBNP, and CD163 and SPON-1 are associated with NYHA class. Based on these results, we conclude that the investigated molecules carry potential for prognostication. They may therefore also potentially contribute to risk assessment and even treatment optimization, such as medication adjustment, earlier referral for interventional procedures (e.g. valvular repair), or circulatory support.

The results of our study shed new light on the relevance of these immune-related biomarkers, which were so far mostly investigated in preclinical studies or with the use of single measurements in clinical settings. We used a unique design with repeated sampling, and we had samples available that

Figure 1 Average temporal evolution of biomarkers in patients during follow-up. Legend: *x*–axis: time remaining to the primary endpoint (for patients who experienced adverse events) or time remaining to the last blood sampling (for patients who remained event free). '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 expressed as *Z*-score of normalized protein expression. Solid red line: Average temporal pattern of biomarker level in patients who reached the primary end point during follow-up. Solid blue line: Average temporal pattern of biomarker level in patients who remained end point free. Betas presented for patients who reached the primary end point (red) and for patients who remained end point free (blue) per one year of follow-up based on linear mixed effects model. Dashed lines: 95% confidence interval. Abbreviations: CD163, scavenger receptor cysteine-rich type 1 protein M130; GRN, granulins; PGLYRP1, peptidoglycan recognition protein 1 (TAG7); SPON1, spondin-1; TFPI, tissue factor pathway inhibitor; TRAP, Tartrate-resistant acid phosphatase type 5.



were drawn closely in time to the endpoint, an approach not applied before in HF patients. We have also applied an advanced statistical method, joint modelling, that provides detailed information on the temporal pattern of the biomarker in association with clinical outcomes. This method is a first step towards a personalized approach for prognostication, as patient-specific biomarker evolution and associated prognosis can easily be derived from the model.[12] Additionally, evaluating repeated measurements instead of baseline values increases the statistical power. Effect estimates obtained from models evaluating repeated measurements may be less prone to attenuation after multivariable adjustment than estimates derived from models evaluating baseline measurements in the same number of patients. This allowed us to demonstrate the associations also after multivariable adjustment in the models that contained repeated measurements, contrary to models that used only baseline values.

Most of the molecules evaluated within our study are involved in the actions of macrophages, and two of them (SPON1 and PGLYRP1) are involved in the response to damage-associated molecular patterns/PAMPs. SPON1 (F-spondin), a molecule associated with adverse clinical outcome, regulates migration and proliferation of macrophages in response to CpG, a pattern typical for bacteria and recognized by toll-like receptor 9 .[16] In human studies, SPON1 was found to correlate negatively with left ventricular ejection fraction[17] and GFR in HF patients, which is in accordance with our results.[18] SPON1 is also an independent predictor of HF and is associated with systolic dysfunction[17] and was identified as a candidate gene for hypertension based on an animal study.[19]

DOI: 10.1002/ehf2.12678

Table 2 Baseline and repeated biomarker measurements in relation to clinical outcome

	Baseline measurement						Repeated measurements					
	Unadjusted		Adjusted for clinical variables[a]		Unadjusted		Adjusted for clinical variables[b]		Adjusted for clinical variables and medication[c]	
Biomarker	HR (95 % CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
CD163	1.52 (1.20–1.92)	<0.001*	1.20 (0.92–1.59)	0.18	2.50 (1.88–3.45)	<0.001*	2.07 (1.47–2.98)	<0.001*	2.60 (1.95; 3.48)	<0.0001*
TRAP	0.77 (0.61–0.98)	0.035	0.85 (0.68–1.06)	0.15	0.60 (0.45–0.80)	<0.001*	0.62 (0.43–0.90)	0.009	0.52 (0.37; 0.74)	0.0003*
GRN	1.36 (1.07–1.72)	0.012	1.09 (0.83–1.43)	0.54	2.511.80–3.59)	<0.001*	2.46 (1.64–3.84)	<0.001*	3.18 (2.33; 4.35)	<0.0001*
TFPI	1.14 (0.90–1.44)	0.286	1.08 (0.83–1.41)	0.74	1.35 (0.98–1.87)	0.059	1.41 (0.98–2.07)	0.059	2.31 (0.91; 1.74)	0.16
SPON1	1.62 (1.32–1.98)	<0.001*	1.28 (0.99–1.65)	0.054	3.77 (2.66–5.52)	<0.001*	3.94 (2.50–6.50)	<0.001*	3.48 (2.42; 4.99)	<0.0001*
PGLYRP1	1.54 (1.23–1.93)	<0.001*	1.19 (0.90–1.58)	0.22	1.91 (1.47–2.51)	<0.001*	1.62 (1.14–2.31)	0.006*	2.20 (1.64; 2.94)	<0.0001*

CD163, scavenger receptor cysteine-rich type 1 protein M130; CI, confidence interval; GRN, granulin; HR, hazard ratio; PGLYRP1, peptidoglycan recognition protein 1 (TAG7); SPON1, spondin-1; TFPI, tissue factor pathway inhibitor; TRAP, tartrate-resistant acid phosphatase type 5.
HRs and 95% CIs are given per 1 SD increase in biomarker expressed in $\log_2$ of normalized protein expression units.
[a]Cox model adjusted for clinical characteristics: age, gender, NYHA class, diabetes mellitus, atrial fibrillation, baseline values of log2 C-reactive protein, systolic blood pressure, and estimated glomerular filtration rate.
[b]Cox and LME model–adjusted for the clinical characteristics: age, gender, NYHA class, diabetes mellitus, atrial fibrillation, baseline values of log2 C-reactive protein, systolic blood pressure, and estimated glomerular filtration rate.
[c]Time-dependent Cox model using fitted biomarker values from the clinical model, adjusted for total daily doses of equivalents of carvedilol, enalapril, furosemide, and spironolactone during follow-up.
*P values significant after Bonferroni correction for multiple testing if $P < 0.008$.

No evidence of its direct influence on cardiomyocytes is available so far, but SPON1 may be associated with several cardiovascular risk factors in this patient population.

Peptidoglycan, a bacterial wall component, belongs to PAMPs recognized by intracellular Nod receptors, and four types of secreted peptidoglycan recognition proteins, PGLYRP-1-4. PGLYRP-1 (TAG7, peptidoglycan recognition protein short) is expressed in neutrophils and macrophages and exhibits bactericidal properties via for example generation of superoxide radicals.[20] Multimers of PGLYRP-1 can also activate a triggering receptor expressed on myeloid cells (triggering receptors expressed on myeloid cells-1) without the presence of peptidoglycan and enhance cytokine release.[21] Further studies are necessary to evaluate its possible association with heart muscle damage and verify whether its plasma levels reflect a causal relationship or just the effect of comorbid atherosclerosis.[22]

CD163 is a member of the scavenger receptor cysteine-rich superfamily and is exclusively expressed in monocytes and macrophages. Levels of soluble CD163 reflect the amount of its membrane-bound form, and soluble CD163 has been identified as a marker of anti-inflammatory M2 macrophage activation.[23] M2 (CD14++CD16++) macrophages are responsible for wound healing, neovascularization, and myofibroblast activation. Their profibrotic action within the myocardium is mediated by osteopontin,[24] which we found to be associated with adverse clinical outcome in our previous investigation.[9] The levels of M2 macrophages are increased in asymptomatic diastolic dysfunction, heart failure with preserved ejection fraction,[25] and HF with reduced ejection fraction.[26] Based on our study, CD163 plasma level increases prior to an adverse clinical event, while the level in event-free patients remains stable. Although the role of the molecule in HF still needs further evaluation in mechanistic studies, we observed for the first time that it is an independent predictor of clinical outcome in a HF population.

TRAP, an iron-containing enzyme, resistant to inhibition by tartrate and activated in acidic conditions, is highly expressed in macrophages and was attributed a role in antigen processing.[27] With regard to cardiovascular disease, an association between TRAP and lower risk of HF hospitalization in patients with chronic kidney disease was observed previously,[18] but it remained unclear what mechanism could lead to this beneficial effect. The authors suggested that this may be a result of its inhibiting effect on osteopontin, which plays an important role in the development of atherosclerosis, but also mediates the profibrotic effect of M2 macrophages mentioned above.[24] The temporal changes of M2 macrophage biomarker CD163 and TRAP levels move in the opposing directions in the present dataset, which may reflect their interplay on the molecular level. We observed that TRAP levels are inversely associated with the occurrence of the PE in the clinically adjusted model that is consistent with the existing literature.

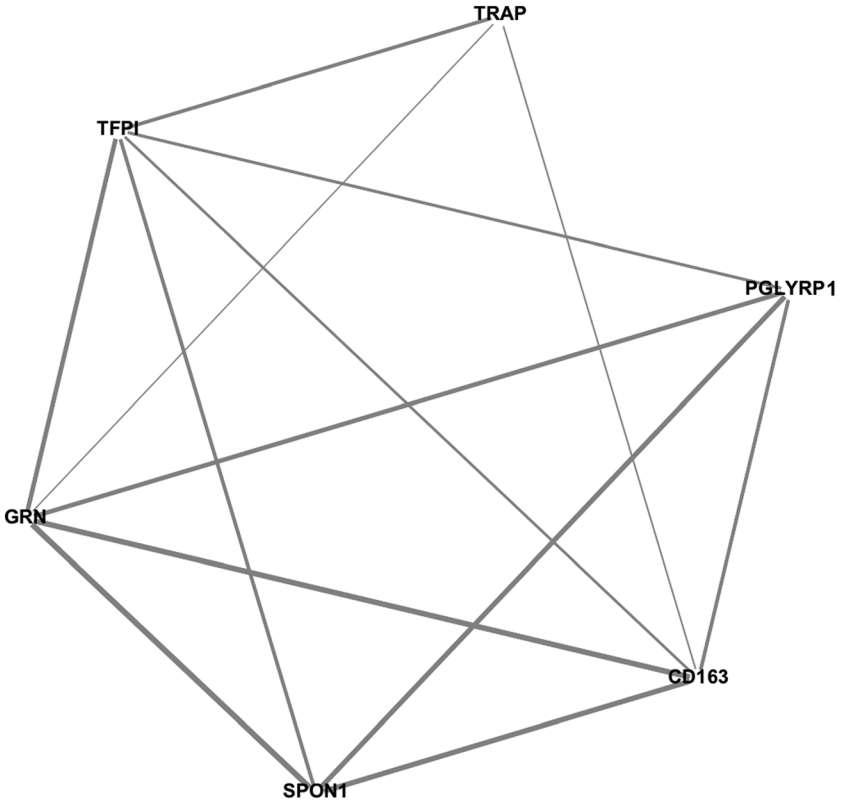
Table 3 Mean change in repeatedly measured biomarkers in relation to baseline characteristics

Independent variable	Dependent variable											
	CD163		TRAP		GRN		SPON1		TFPI		PGLYRP1	
	B (95% CI)	*P* value	*B* (95% CI)	*P* value	*B* (95% CI)	*P* value	*B* (95% CI)	*P* value	*B* (95% CI)	*P* value	*B* (95% CI)	*P* value
Time (months)	NS		−0.10 (−0.04; −0.16)	0.002	NS		NS		NS		0.07 (0.02; 0.12)	0.005
Age (per 10 years)	NS		0.14 (0.03; 0.25)	0.011	NS		NS		−0.13 (−0.23; −0.02)	0.024	NS	
Male gender	NS		NS		−0.36 (−0.58; −0.13)	0.001	NS		NS		NS	
NYHA class	0.16 (0.02; 0.31)	0.027	NS		NS		0.14 (0.03; 0.25)	0.012	NS		NS	
DM	0.37 (0.14; 0.61)	0.002	NS		0.30 (−0.09; 0.52)	0.006	0.31 (0.13; 0.49)	<0.001	NS		NS	
AF	0.23 (0.02; 0.45)	0.035	NS		0.26 (0.06; 0.46)	0.012	0.34 (0.13; 0.49)	<0.001	NS		NS	
SBP (per 10 mmHg)	NS		NS		NS		NS		NS		NS	
eGFR (per 20 mL/BSA)	NS		NS		−0.14 (−0.24; −0.04)	0.005	−0.10 (−0.18; −0.02)	0.011	−0.18 (−0.28; −0.07)	0.001	−0.22 (−0.32; −0.13)	<0.001
NT-proBNP (per doubling)	NS		−0.09 (−0.16; −0.02)	0.014	0.004 (−0.05; 0.07)	0.023	0.13 (0.07; 0.18)	<0.001	−0.04 (−0.10; 0.03)	0.002	0.05 (−0.01; 0;11)	<0.001
Hs-TnT (per doubling)	NS		NS		NS		NS		NS		0.19 (0.08; 0.31)	<0.001
CRP (per doubling)	0.007 (0.001;0.011)	0.003	NS		0.007 (0.003; 0.011)	<0.001	0.005 (0.002; 0.009)	<0.001	NS		NS	
Carvedilol equivalent (per 50 mg)	NS		NS		NS		NS		NS		NS	
Enalapril equivalent (per 40 mg)	NS		NS		NS		NS		NS		NS	
Furosemide equivalent (per 40 mg)	NS		NS		NS		0.06 (0.02; 0.09)	0.004	NS		NS	
Spironolactone equivalent (per 25 mg)	−0.23 (−0.41; −0.06)	0.008	NS		−0.27 (−0.43; −0.11)	0.001	−0.17 (−0.30; −0.03)	0.015	−0.27 (−0.46; −0.10)	0.003	−0.17 (−0.326; −0.006)	0.042

AF, atrial fibrillation; BSA, body surface area; CRP, C-reactive protein; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; Hs-TnT, high-sensitivity troponin T; NS, not significant; NYHA, New York Heart Association; SBP, systolic blood pressure.

The effects are given as adjusted *B* (95% CI). Independent variables are the patients' baseline characteristics, dependent variables are Z-scores of biomarkers as measured in normalized protein expression units on the $\log_2$ scale. This method allows a direct comparison of the effects on different biomarkers. All betas are adjusted for patients' age, sex, body mass index, DM, AF, baseline NYHA class, SBP, eGFR, N-terminal pro BNP levels, Hs-TnT levels, and equivalent doses of carvedilol, enalapril, furosemide, and spironolactone. Only the associations with significance level of $P < 0.05$ are presented.

Figure 2 Network analysis of biomarkers depicting intermarker correlations.The thickness of the line between the biomarkers represents the correlation coefficient (presented only if $P < 0.05$); a thicker line represents higher coefficients. Abbreviations: CD163, scavenger receptor cysteine-rich type 1 protein M130; GRN, granulins; PGLYRP1, peptidoglycan recognition protein 1 (TAG7); SPON1, spondin-1; TFPI, tissue factor pathway inhibitor; TRAP, tartrate-resistant acid phosphatase type 5.



We also observed a positive association between GRN and the PE. The assay we used is based on an antibody that binds with progranulin (PGRN), a precursor protein that is later cleaved into different GRN but also with GRN themselves. Progranulin (proepithelin, granulin/epithelin precursor), a pleiotropic growth factor, has not been investigated in heart failure patients so far. It is highly expressed in epithelial cells, neurons, and macrophages but also to a lesser extent in many other types of cells.[28] So far its role in malignancies, neurodegenerative and autoimmune diseases has been elucidated. Although multiple immunity-related actions, both pro-inflammatory and anti-inflammatory, of progranulin have been described, its role is not fully understood so far. For instance, progranulin competes with the strong pro-inflammatory cytokine tumour necrosis factor-α by binding to its receptors type 1 and 2 and diminishes inflammation.[29] On the other hand, progranulin cleavage products, which include several types of GRN, may increase tumour necrosis factor-α and IL-1β levels and therefore augment inflammation.[30] Unfortunately, in contrast to the abundance of data on progranulin, there is a lack of evidence on specific GRN in the literature. With the method used, we also cannot discriminate between the impact of progranulin and its cleavage products. Our results may be therefore viewed as hypothesis generating and indicate a need for further studies. The equivocal role of progranulin is further illustrated by the fact that it exerted a stimulatory effect on insulin resistance,[31] which goes in line with our observation on higher GRN levels in diabetic patients, but there is also evidence of its beneficial role in reducing renal and cardiac damage in animal models.[32,33]

Together with the immune system biomarkers, we evaluated a marker of tissue response to the activation of the immune system. It has previously been reported that the expression of anticoagulant TFPI is increased in response to the tumour necrosis factor-α driven hypercoagulable state within the myocardium.[34] However, we did not observe an association of TFPI with clinical outcome in our patients.

Apart from their potential for prognostication and even treatment guidance and the mechanistic insights they provide, the investigated molecules may be also interesting as therapeutic targets. Preclinical studies on antagonists of innate immune receptors (TLR2 and toll-like receptors type 4) and innate immune signalling pathways (myeloid differentiation primary response 88, interleukin-1 receptor-associated kinase 1, interleukin-1 receptor-associated kinase 4, and NLRP3) have shown promising results, including a reduction

DOI: 10.1002/ehf2.12678

in adverse left ventricular remodelling after acute myocardial infarction.[1] In this light, further evaluation of these innate immunity-related molecules as therapeutic targets may be of interest. We have shown that the functions of some of the investigated molecules (TRAP, osteopontin, and M2 macrophage biomarker CD163) are linked to each other. Such a set of interrelated biomarkers could provide a tool for 'mechanistic phenotyping', that could potentially help us distinguish a subgroup of patients who would benefit from anti-inflammatory treatment in a more reliable way than a single biomarker. Such an approach has recently been applied with several fibrotic biomarkers used for evaluation of response to spironolactone in a *post hoc* analysis of the Treatment of Preserved Cardiac Function Heart Failure With an Aldosterone Antagonist trial.[35] Moreover, evaluation of a possible extra-cardiac origin of the investigated molecules may shed some new light on their role, based on our observation that levels of some of these biomarkers are higher in patients with DM (CD163, GRN, and SPON1) and correlate inversely with renal function (GRN, SPON1, TFPI, and PGLYRP1). Tissues of interest could include adipose tissue, kidneys, or lungs.

Some aspects of this study warrant consideration. First, this cohort consisted mainly of patients with HF with reduced ejection fraction patients, and the results can therefore not be extrapolated to CHF patients with preserved LVEF. Second, concomitant measurement of monocyte subsets could have yielded more information on the role of this selected group of molecules. Third, the method we used does not allow us to draw separate conclusions on progranulin and GRN.

In conclusion, the temporal evolution of innate immune system biomarkers CD163, TRAP, GRN, SPON1, and PGLYRP1 is associated with adverse clinical endpoints in a CHF population independently of traditional risk factors. These findings suggest that these markers may be used to identify HF patients at increased risk of an adverse disease course.

Conflict of interest

Dominika Klimczak-Tomaniak, Elke Bouwens, Anne-Sophie Schuurman, K. Martijn Akkerhuis, Alina Constantinescu, Jasper Brugts, B. Daan Westenbrink, Jan van Ramshorst, Tjeerd Germans, Leszek Pączek, Victor Umans, Eric Boersma, and Isabella Kardys declare no conflict of interest.

Funding

The Bio-SHiFT study was supported by the Jaap Schouten Foundation (Rotterdam, the Netherlands) and the Noordwest Academie (Alkmaar, the Netherlands).

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Appendix S1 - Supporting Information
Table S1. Univariable Cox model of baseline characteristics of the study population.
Table S2. Medication dosage equivalents.
Table S3. Pearson correlation coefficients between the biomarkers based on their first measurement.
Table S4. Molecular function of biomarkers and their involvement in biological processes based on panther database
Table S5. Baseline and repeated biomarker measurements in relation to the primary outcome in the subgroup of patients with HF-REF (N = 250, N of events = 66)
Table S6. Associations of the biomarkers with the secondary endpoint consisting of heart failure hospitalization and cardiovascular death (N = 263, no. of events = 65)
Table S7. Associations of the biomarkers with the PE in patients with ischemic etiology of heart failure (N = 117, N of events = 36).
Table S8. Associations of the biomarkers with the PE in patients with dilated cardiomyopathy (N = 49, N of events = 9).
Figure S1. *Inclusion and exclusion criteria.*

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