ORIGINAL RESEARCH

Thymosin Beta-4 Is Elevated in Women With Heart Failure With Preserved Ejection Fraction

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***Background*—**Thymosin beta-4 (TB4) is an X-linked gene product with cardioprotective properties. Little is known about plasma concentration of TB4 in heart failure (HF), and its relationship with other cardiovascular biomarkers. We sought to evaluate circulating TB4 in HF patients with preserved (HFpEF) or reduced (HFrEF) ejection fraction compared to non-HF controls.

***Methods and Results*—**TB4 was measured using a liquid chromatography and mass spectrometry assay in age- and sex-matched HFpEF (n=219), HFrEF (n=219) patients, and controls (n=219) from a prospective nationwide study. Additionally, a 92-marker multiplex proximity extension assay was measured to identify biomarker covariates. Compared with controls, plasma TB4 was elevated in HFpEF (985 [421–1723] ng/mL versus 1401 [720–2379] ng/mL, $P<0.001$), but not in HFrEF (1106 [556–1955] ng/mL, $P=0.642$). Stratifying by sex, only women (1623 [1040–2625] ng/mL versus 942 [386–1891] ng/mL, $P<0.001$), but not men (1238.5 [586–1967] ng/mL versus 1004 [451–1538] ng/mL, $P=1.0$), had significantly elevated TB4 in the setting of HFpEF. Adjusted for New York Heart Association class, N-terminal pro B-type natriuretic peptide, age, and myocardial infarction, hazard ratio to all-cause mortality is significantly higher in women with elevated TB4 (1.668, $P=0.036$), but not in men (0.791, $P=0.456$) with HF. TB4 is strongly correlated with a cluster of 7 markers from the proximity extension assay panel, which are either X-linked, regulated by sex hormones, or involved with NF-κB signaling.

***Conclusions*—**We show that plasma TB4 is elevated in women with HFpEF and has prognostic information. Because TB4 can preserve EF in animal studies of cardiac injury, the relation of endogenous, circulating TB4 to X chromosome biology and differential outcomes in female heart disease warrants further study. **(*J Am Heart Assoc*. 2017;6:e005586. DOI: 10.1161/JAHA.117.005586.)**

Key Words: biomarker • cardiac biomarkers • CD40/CD40L • heart failure • liquid chromatography-mass spectrometry • proximity extension assay • thymosin beta-4

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Accompanying Tables S1 through S4 and Figures S1 through S3 are available at http://jaha.ahajournals.org/content/6/6/e005586.full#sec-23

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Sex differences in the epidemiology and pathophysiology of heart failure (HF) are well known.[1,2] Women generally experience symptoms later in life, are less likely than men to present with ischemic disease, and predominate in the subset of HF with preserved ejection fraction (HFpEF).[3] However, unlike HF with reduced ejection fraction (HFrEF), clinical diagnosis of HFpEF remains challenging and controversial.[4] Established biomarkers such as N-terminal pro B-type natriuretic peptide (NT-proBNP) and high-sensitivity troponin T (hsTnT) are not specific for HFpEF.[5,6] The need for insight into HFpEF physiology is particularly acute as there are no therapeutic strategies proven to alter disease progression.[6]

Mature thymosin beta-4 (TB4) is a highly conserved 43-amino acid peptide.[7,8] When endogenous levels of TB4 are supplemented with exogenous delivery, animal models of cardiac injury show dramatic reduction in fibrosis,[9,10] increase in recruited stem cells,[11,12] and preservation of EF.[10,13] The underlying mechanism of TB4 cardioprotection has been attributed to increase in stem cell mobilization[11,12] and angiogenesis as well as reduction in inflammation and apoptosis.[10,14,15] In cardiomyocyte culture, TB4 can prevent angiotensin II–induced hypertrophy,[16] activate integrin-linked kinase,[10] and upregulate copper/zinc superoxide dismutase and catalase to provide resistance to oxidative stress.[17]

Clinical studies of circulating TB4 have been limited. A study of TB4 in HF patients undergoing cardiac stem cell infusion showed that TB4 was selectively elevated in patients who experienced symptomatic improvement.[18] Two other studies have correlated plasma TB4 level to the development of collateralization in patients with severe coronary artery disease.[19,20]

In this study, we quantified endogenous plasma levels of TB4 in 657 age- and sex-matched HF patients and controls, using liquid chromatography and mass spectrometry (LCMS). Although TB4 is an established cardioprotective molecule, little is known about the clinical significance of circulating TB4 in HF, including its source and regulation. To understand the broader soluble biomarker context of TB4 and provide hypothesis generation, we correlated TB4 measurements with a panel of 92 other cardiac biomarkers.

Methods

Patients and Study Design

The study cohort (n=657) was prospectively formed to comprise 219 non-HF controls, 219 HFpEF cases, and 219 patients with HFrEF selected for demographic comparability (ie, ethnicity, sex, and age). Patients with HF were chosen from the Singapore Heart Failure Outcomes and Phenotypes cohort[21] and control participants from the Singapore Longitudinal Aging Study.[22] Singapore Heart Failure Outcomes patients were either those admitted with primary diagnosis of acute decompensated HF according to European Society of Cardiology criteria,[23] or those attending an outpatient clinic for management of HF with a history of an acute decompensated HF admission within the previous 6 months. Patients with HF attributable to infiltrative or congenital heart disease were excluded. Non-HF control participants subselected from the Singapore Longitudinal Aging Study cohort were free of coronary artery disease or HF by history and clinical and echocardiographic examination. Both Singapore Heart Failure Outcomes and Singapore Longitudinal Aging Study studies were approved by the ethics review board of each participating institution and complied with the Declaration of Helsinki. Informed and signed consent was provided by all participants in this study. Importantly, blood sampling and other assessments were all undertaken with patients in the stable compensated state (ie, shortly before discharge or during outpatient consultation). Blood samples were taken into EDTA tubes with prompt separation by centrifuging at 1485 g with cooling, and the plasma stored at −80°C pending assay.

Measurement of Biomarkers

Measurement of plasma NT-proBNP and hsTnT has been previously described.[24] NT-proBNP and hsTnT concentrations were determined by electro-chemiluminescence immunoassay using the NT-proBNP II and troponin T high-sensitivity assays, respectively, on an ELECSYS Cobas e411 immunoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). The ranges of measurement for NT-proBNP and hsTnT assays were 5 to 35 000 pg/mL and 3 to 10 000 pg/mL, respectively. Mean concentrations and interassay coefficient of variation of samples used for quality control, presented as mean (coefficient of variation), were established in-house. Low-concentration NT-proBNP and hsTnT quality control samples averaged at 141 pg/mL (3.38%) and 26.7 pg/mL (6.66%), respectively; high NT-proBNP and hsTnT samples averaged at 4759 pg/mL (4.03%) and 2090 pg/mL (4.06%), respectively.

A multiplex biomarker panel based on proximity extension technology was also utilized to get relative quantification on a panel of 92 biomarkers (Proseek Multiplex CVD I, Olink Proteomics AB, Uppsala, Sweden).

LCMS/MS Method for TB4 Quantification

We developed and validated an LCMS method for TB4 assay. Liquid chromatographic separation of TB4 was carried out on an Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, CA) with Kinetex 2.6-μm XB-C18 100 Å 100×2.1-mm UPLC column (Phenomenex, Torrance, CA)

Table 1. Demographics and Clinical Characteristics of Cohort

	Controls (n=219)	HFpEF (n=219)	HFrEF (n=219)	*P*-Value
Clinical characteristics				
Age, y	65.0±9.0	68.2±11.2	64.7±11.2	0.001
Sex				
Male	106 (48.4%)	104 (47.5%)	117 (53.4%)	0.409
Female	113 (51.6%)	115 (52.5%)	102 (46.6%)	
Ethnicity				
Chinese	148 (67.6%)	141 (64.4%)	148 (67.6%)	0.656
Malay	51 (23.3%)	56 (25.6%)	50 (22.8%)	
Indian	20 (9.1%)	19 (8.7%)	20 (9.1%)	
Other	0 (0.0%)	3 (1.4%)	1 (0.5%)	
BMI, kg/m^2	25.0±3.9	27.6±5.7	25.5±5.4	<0.001
Heart rate, beats/min	66.8±9.4	71.8±13.4	76.4±14.0	<0.001
Systolic BP, mm Hg	138.4±20.1	132.1±20.6	124.3±22.2	<0.001
Diastolic BP, mm Hg	76.1±10.8	69.3±11.4	70.1±11.9	<0.001
NYHA Class				
I	210 (95.9%)	51 (24.9%)	46 (21.5%)	<0.001
II	9 (4.1%)	124 (60.5%)	129 (60.3%)	
III & IV	0 (0.0%)	30 (14.6%)	39 (18.2%)	
Ischemic etiology	…	87 (39.7%)	145 (66.2%)	<0.001
Coronary artery disease	…	68 (34.2%)	120 (57.4%)	<0.001
Hypertension	91 (41.7%)	177 (85.9%)	155 (71.4%)	<0.001
Diabetes mellitus	27 (12.4%)	121 (58.5%)	131 (60.1%)	<0.001
Atrial fibrillation/flutter	3 (1.4%)	64 (30.8%)	50 (23.2%)	<0.001
Peripheral vascular disease	0 (0.0%)	3 (1.5%)	10 (4.6%)	0.002
Cancer	7 (3.3%)	5 (2.5%)	11 (5.1%)	0.330
History of smoking				
Nonsmoker	173 (79.7%)	151 (73.0%)	137 (63.1%)	0.004
Ex-smoker	25 (11.5%)	33 (15.9%)	49 (22.6%)	
Current smoker	19 (8.8%)	23 (11.1%)	31 (14.3%)	
Death within 2 y	3 (1.4%)	24 (11.0%)	36 (16.4%)	<0.001
HF re-hospitalization within 2 y	0 (0%)	61 (27.9%)	77 (35.2%)	<0.001
HF rehospitalization OR death within 2 y	3 (1.4%)	79 (36.1%)	93 (42.5%)	<0.001
Laboratory values				
Sodium, mmol/L	139±5	136±15	138±4	<0.001
Hemoglobin, g/dL	13.8±1.6	11.8±2.1	12.5±1.9	<0.001
White blood cells, $10^3/\mu L$	6.0±1.6	8.9±3.0	8.2±2.6	<0.001
Albumin, g/L	36.9±14.4	25.0±15.3	23.9±15.9	<0.001
Creatinine, μmol/L	70±22	119±58	117±50	<0.001
eGFR, mL/min	98.6±27.7	60.8±30.5	60.2±23.7	<0.001
NT-proBNP, pg/mL	76 (41–131)	919 (330–2315)	2880 (1299–7134)	<0.001
hsTnT, pg/mL	8 (6–11)	22 (14–41)	27 (17–51)	<0.001
TB4, ng/mL	985 (421–1723)	1401 (720–2379)	1106 (556–1955)	<0.001

Continued

Table 1. Continued

	Controls (n=219)	HFpEF (n=219)	HFrEF (n=219)	*P*-Value
Medications				
Diuretic	···	181 (82.7%)	195 (89.0%)	0.055
ACE inhibitor/ARB	···	126 (57.5%)	158 (72.2%)	0.001
β-Blocker	···	178 (81.3%)	189 (86.3%)	0.154
Aldosterone antagonist	···	27 (12.3%)	96 (43.8%)	<0.001
Digoxin	···	28 (12.8%)	66 (30.1%)	<0.001
Statin	···	189 (86.3%)	180 (82.2%)	0.238
Echocardiographic data				
LVEF, %	65±4	59±6	31±10	<0.001
Mitral E/e′ ratio	11.2±3.8	17.2±9.6	22.3±11.2	<0.001

Values are mean (±SD), median (interquartile range), or n (%). ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; BMI, body mass index; BP, blood pressure; eGFR, estimate glomerular filtration rate; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; hsTnT, high sensitivity troponin T; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro B-type natriuretic peptide; NYHA, New York Heart Association (functional class); OR, odds ratio; TB4, thymosin beta-4.

maintained at 30°C. The aqueous solvent used was 0.1% formic acid in water and organic solvent was 0.1% formic acid in acetonitrile. A linear LC gradient was set up with percentage of organic solvent as follows: 5% at 0 minute, 10% at 2.5 minutes, 30% at 3.5 minutes, and 95% between 4 and 4.5 minutes, with flow rate of 0.6 mL/min. The sample injection volume was 10 μL. For mass detection, the LC eluent is connected online to an Agilent 6495 Triple Quadrupole MS system (Agilent Technologies) operated with the electrospray source in positive ionization mode. The electrospray ionization source conditions were as follows: capillary voltage of 4.5 kV, nozzle voltage of 500 V, iFunnel parameter high/low pressure RF of 200 V, nebulizer pressure of 60 psi, gas temperature of 350°C, sheath gas temperature of 400°C, and sheath gas flow of 12 L/min.

Synthetic TB4 (Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPL PSKETIEQEKQAGES-acid) (purity >95%) (Cambridge Research Biochemical, Billingham, Cleveland, UK) and a heavy isotope version (Ac-SDK-[U-13C5,15n-Pro]-DMAEIEKFDKSKLKKTETQE Kn-[U-13C5,15n-Pro]-L-[U-13C5,15n-Pro]-SKETIEQEKQAGES-acid) (purity >95%) (Cambridge Research Biochemical) were used as standards for development of multiple reaction monitoring methods for quantification. The multiple reaction monitoring used for TB4 and its heavy isotope standard were 709.3→810 with collision energy of 15 eV, and 713→658.4 with collision energy of 18 eV, respectively. TB4 quantification was carried out against a calibration curve serially diluted in rabbit plasma (Biowest, Nuaillé, France) at concentrations of 2083, 1042, 521, 260, 130, and 65 ng/mL (Figure S1). For quality control, rabbit plasma spiked with 1700, 700, and 100 ng/mL of TB4 were also run in each LCMS/MS batch. The mean (coefficient of variation) measured for each quality control were 1809 ng/mL (5.0%), 660 ng/mL (3.7%), and 116 ng/mL (2.3%), respectively. Where TB4 levels exceeded the quantification range, plasma sample was diluted using rabbit plasma before requantification.

Sample Preparation for LCMS/MS

Human plasma samples (50 μL) were aliquoted into a 96-well plate, then spiked with 100 μL of 1 μg/mL TB4 heavy isotope standard. After treating with 150 μL of acetonitrile, the plate was mixed on a shaker at 1000 rpm/min for 5 minutes, then spun down at 4000*g* for 20 minutes at 4°C. Fifty microliters of the supernatant was carefully transferred to a 96-microwell plate and further diluted with 50 μL of ultrapure water containing 0.2% formic acid. The 96-microwell plate containing the diluted filtrate was then loaded into the auto-sampler for analysis by LCMS/MS. Ion counts were then normalized against that of the heavy isotope standard, before using the standard curve for quantification.

Statistical Analyses

All statistical tests were 2-sided and conducted at 5% level of significance with Stata MP V14 (StataCorp LLC, College Station, TX) unless otherwise stated. Categorical variables are presented as numbers (percent) and continuous variables as mean±SD or median (interquartile range) as appropriate. For intergroup comparisons, Kruskal–Wallis test or χ^2 test was applied as appropriate. To identify independent predictors of plasma biomarker concentrations, clinical characteristics were first analyzed as univariates. The significant variables

Table 2. Pairwise Comparison of Plasma TB4 Levels (ng/mL), Stratified by Sex and HF Status

Comparison Median (IQR)		Difference in Median	*P*-Value	*P*-Value (Corrected)*
Control 985 (421–1723)	HF 1265 (638–2146)	280	<0.001†	0.002†
	HFpEF 1401 (720–2379)	416	<0.001†	<0.001†
	HFrEF 1106 (556–1955)	121	0.040†	0.642
HFrEF	HFpEF	295	0.002†	0.037†
Women control 942 (386–1891)	Women HF 1512 (939–2344)	570	<0.001†	<0.001†
	Women HFpEF 1623 (1040–2625)	681	<0.001†	<0.001†
	Women HFrEF 1278 (757–2154)	332	0.006†	0.102
Women HFrEF	Women HFpEF	350	0.028†	0.448
Men control 1004 (451–1538)	Men HF 1030 (513–1858)	26	0.285	1.000
	Men HFpEF 1239 (586–1967)	235	0.066	1.000
	Men HFrEF 938 (451–1822)	66	0.925	1.000
Men HFrEF	Men HFpEF	301	0.069	1.000
Men control	Women control	62	0.937	1.000
Men HF	Women HF	482	<0.001†	<0.001†
Men HFpEF	Women HFpEF	385	0.001†	0.019†
Men HFrEF	Women HFrEF	336	0.004†	0.056

P-values were corrected with Bonferroni correction. HF indicates heart failure; HFpEF indicates heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; IQR, interquartile range; TB4, thymosin beta-4.
*Bonferroni correction for 16 comparisons.
†Statistically significant.

from the initial univariate analysis are then entered into a multivariate linear regression model to be estimated by the least-squares method. A final model is then derived by using backward elimination with a cutoff *P*-value of 0.05. The ability of TB4 and NT-proBNP to discriminate HF (and its subgroups HFpEF and HFrEF) from control was evaluated by receiver operating curve analysis.

Assessment of the prognostic performance (for time to 2-year all-cause mortality) of TB4 was conducted by comparing Kaplan–Meier survival functions using log-rank test, and generalized structure equation modeling (gSEM), a model-building framework that allows for complex data interaction to be accommodated with ease.[25,26] Weibull distribution with log link were used to handle time to all-cause mortality, and "robust" standard errors correction was applied in anticipation of potential bias caused by some outliers.

Pairwise correlation of Ln TB4, Ln NTproBNP, Ln hsTnT, and the panel of 92 biomarkers was done using the "matpwcorr" module on STATA with Bonferroni correction. Significant correlates of Ln TB4 were then ranked by Pearson correlation coefficient. Graphical representation of the correlation was generated using the "corrplot" package in RStudio (Version 0.99.902) with "hclust" ordering.

Results

Cohort Characteristics

Cohort characteristics are shown in Table 1. In agreement with previous reports, in our cohort, hypertension was more common in HFpEF,[4] ischemic etiology more common in HFrEF, and NT-proBNP levels were highest in HFrEF.[27] Median level (interquartile values) of plasma TB4 was 985 (421–1723) ng/mL in controls, 1401 (720–2379) ng/mL in HFpEF, and 1106 (556–1955) ng/mL in HFrEF.

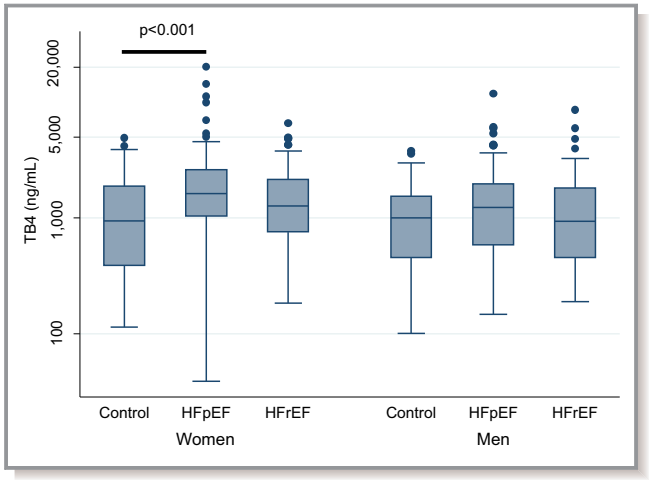

Figure 1. Tukey plot of plasma thymosin beta-4 (TB4) levels in control, HFpEF, and HFrEF patients, stratified by sex. Elevation of plasma TB4 is significant (P<0.001) in female patients with HFpEF. HFpEF indicates patients with heart failure with preserved ejection fraction; HFrEF, patients with heart failure with reduced ejection fraction.

Pairwise Comparison of TB4 Levels by Sex and HF Status

We performed pairwise comparison of TB4 levels by HF status and sex (Table 2). The elevation of TB4 in HF overall compared with control was significant (1265 [638–2146] versus 985 [421–1723], P=0.002). This significance was predominantly driven by the HFpEF subset of HF (1401 [720–2379] versus 985 [421–1723], P<0.001); HFrEF values did not differ significantly from controls (1106 [556–1955] versus 985 [421–1723], P=0.642).

When patients with HFpEF were further stratified by sex, statistical significance was found to be specifically driven by women with HFpEF in comparison to controls (1623 [1040–2625] ng/mL versus 942 [386–1891] ng/mL, P<0.001). No similar association was found in male patients (1239 [586–1967] ng/mL versus 1004 [451–1538] ng/mL, P=1.000). These stratified data are further presented as a boxplot in Figure 1. The distributions of TB4 within male and female HF populations were similar with an interquartile range for male patients with HF of 1345 ng/mL versus 1405 ng/mL for female patients with HF.

Table 3. Independent Correlates of TB4 in Patients With HF From Univariate Analysis Followed by Multivariate Analysis With Backward Elimination

Ln TB4, n=365, Adjusted R^2=0.126			
Clinical Variables	Coefficient	95% CI	P-Value
Women vs men	0.282	0.116 to 0.448	0.001
Peripheral vascular disease	0.612	0.173 to 1.051	0.006
Diastolic BP, mm Hg	−0.008	−0.015 to −0.000	0.041
NYHA Class			
2 vs 1	0.249	−0.044 to 0.455	0.018
3&4 vs 1	0.415	0.149 to 0.681	0.002
hsTnT*, pg/mL	0.106	0.003 to 0.209	0.043
LVEF in %	0.009	0.004 to 0.014	<0.001

BP indicates blood pressure; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association (functional class); hsTnT, high sensitivity troponin T; TB4, thymosin beta-4.
*Natural logarithm of hsTnT.

Clinical Correlates of TB4

To identify clinical correlates of plasma TB4 in the HF cohort, we examined 33 clinical variables including those previously identified to associate with mortality in HF, using a univariate linear regression (Table S1). Statistically significant variates were then included in a multivariate model for refinement by backward elimination. The 6 significant correlates of TB4 included in the final multivariate model (Table 3) were the following: sex (TB4 is elevated in women), peripheral vascular disease (elevated), diastolic blood pressure (inversely related), New York Heart Association (NYHA) class (elevated in higher classes), hsTnT levels (positively correlated), and left ventricular ejection fraction (positively correlated). Notably, Ln TB4 did not significantly correlate with Ln NT-proBNP. Established biomarkers NT-proBNP and hsTnT were also subjected to the same multivariate analysis (Tables S2 and S3) but only TB4 was significantly higher in women. As expected, hsTnT was significantly higher in men.

Biomarker Correlates of TB4

As TB4 levels seemed to be sex specific and were independent of NT-proBNP, we attempted to discover other possible biomarkers indicative of candidate pathophysiological pathways that may correlate with TB4. TB4 correlated best with CD40 ligand/CD154 (R=0.755, P<0.001), pro-epidermal growth factor (R=0.744, P<0.001), heat shock 27-kDa protein —HSP27 (R=0.739, P<0.001), NF-kappa-B essential modulator (R=0.733, P<0.001), proto-oncogene tyrosine-protein kinase Src (R=0.730, P<0.001), integrin beta 1 binding protein 2 (melusin) (R=0.712, P<0.001), and Nicotinamide adenine dinucleotide-dependent deacetylase sirtuin-2 (R=0.708, P<0.001) (Figure 2). A full list of the 92 biomarkers and their correlation coefficient with TB4 are provided in Table S4. A visualization of the correlation matrix ordered with hierarchal clustering is shown in Figure S2, and the scatter plot of the well-correlated biomarkers is shown in Figure 2.

Diagnostic Performance of TB4

As TB4 was significantly elevated in female patients with HFpEF, we hypothesized that TB4 may have diagnostic utility

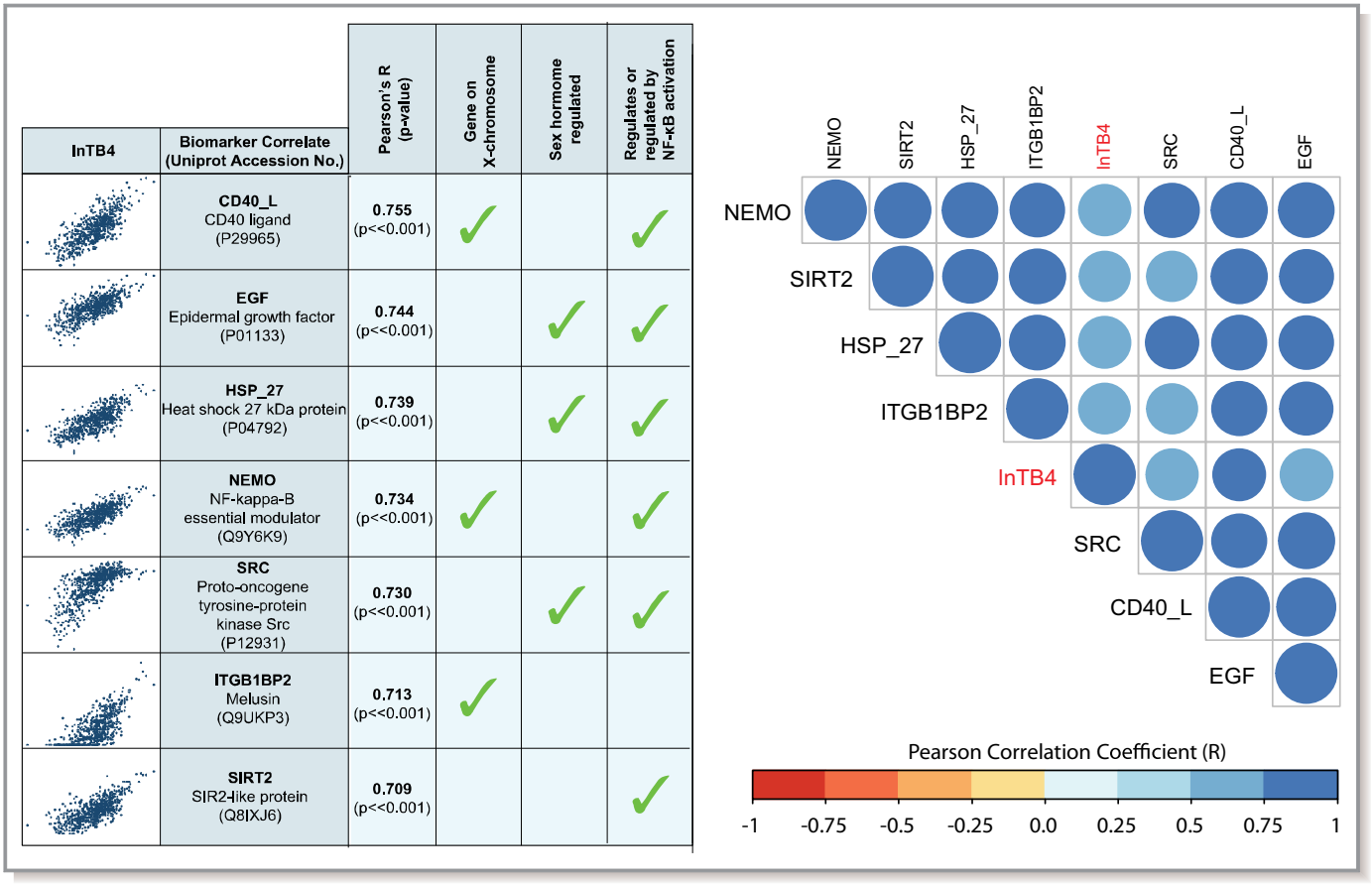

Figure 2. Biomarker correlates of TB4. TB4 clusters with a group of co-correlating biomarkers that are either encoded by genes on X-chromosome or regulated by sex hormone. TB4, thymosin beta-4.

for HFpEF in women. We evaluated the performance of TB4 in discriminating between HFpEF and control women by receiver operating curve analysis, which yielded an area under curve of 0.679 (95% CI: 0.609–0.748; $P<0.001$). The corresponding area under curve for NT-proBNP was 0.908 (0.867–0.949). Consistent with TB4 being significantly elevated selectively in women, the areas under curve were nondiscriminatory in male patients (Figure 3).

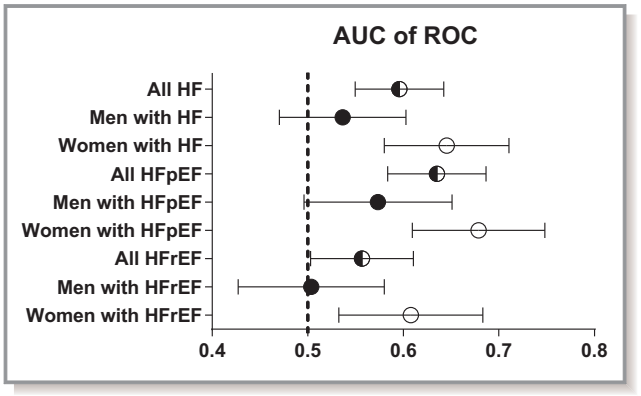

Figure 3. ROC values of TB4 for selected HF (heart failure) diagnosis scenarios. Error bar denotes 95% CI. TB4 only has significant diagnostic value in scenarios with women. Both sexes (◐), men only (●), and women only (○). AUC indicates area under curve; HFpEF patients with heart failure with preserved ejection fraction; HFrEF, patients with heart failure with reduced ejection fraction; ROC, receiver operating curve; TB4, thymosin beta-4.

Kaplan–Meier Curves for All-Cause Mortality

Over 2 years' follow-up among patients with HF, there were a total of 60 deaths (33 in men, 27 in women). To investigate whether TB4 has prognostic value, we grouped HF patients by their TB4 levels into the top tertile and lower 2 tertiles. Kaplan–Meier survival curves were constructed for 2-year all-cause mortality (Figure 4). When all HF patients were considered (cutoff value=1769 ng/mL, $P=0.1638$), or analysis was confined to men (cutoff value=1452 ng/mL, $P=0.9910$), the difference in survival functions between the top and bottom 2 tertiles was not statistically significant. In contrast, for female patients with HF, the difference in survival functions was significant (cutoff value=2000 ng/mL, $P=0.0128$). Synergism between NT-proBNP and TB4 was assessed by splitting patients into 4 groups based on whether they were above or below

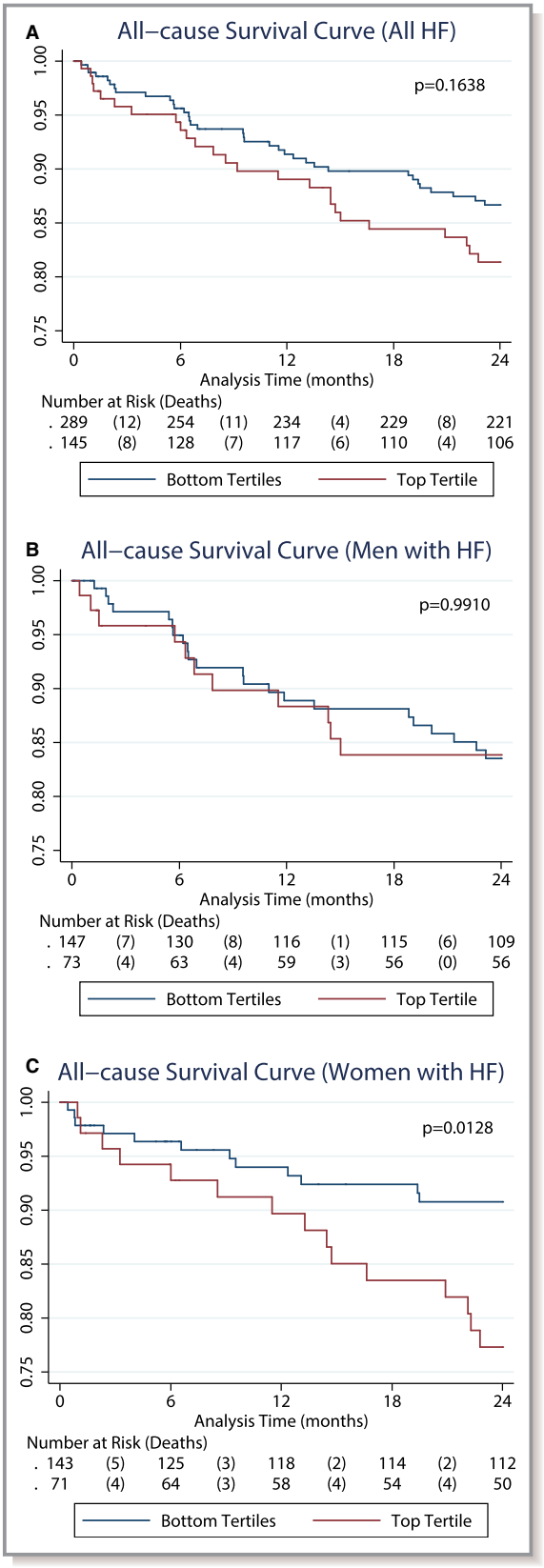

Figure 4. All-cause Kaplan–Meier survival curves segmented based on TB4 levels. Two-year all-cause Kaplan–Meier survival curves for (A) all heart failure (HF), (B) only male HF, and (C) only female HF. TB4, thymosin beta-4.

Table 4. gSEM Modeling for Time to All-Cause Mortality for All Sexes in Initial Model

Time to Mortality	Adjusted Hazard Ratio	95% CI	*P*-Value
All HF patients, n=350, 48 deaths			
Ln NT-proBNP	1.675	1.159 to 2.421	0.006*
Ln TB4	0.927	0.592 to 1.453	0.742
HFrEF vs HFpEF	0.960	0.465 to 1.979	0.912
Age, y	1.056	1.014 to 1.099	0.008*
NYHA class			
2 vs 1	1.463	0.410 to 5.225	0.558
3&4 vs 1	4.492	1.250 to 16.148	0.021*
Ischemic etiology	0.840	0.336 to 2.104	0.710
Diabetes mellitus	1.287	0.512 to 3.240	0.591
Atrial fibrillation	1.039	0.548 to 1.970	0.906
Sodium	0.999	0.980 to 1.019	0.929
Mitral E/e′	1.009	0.987 to 1.032	0.419
eGFR	1.007	0.984 to 1.030	0.540
Coronary artery disease	0.408	0.118 to 1.409	0.156
Myocardial infarction	3.069	1.064 to 8.850	0.038*
Hypertension	0.766	0.319 to 1.839	0.550
Heart rate	1.021	0.997 to 1.044	0.082
Systolic blood pressure	0.990	0.969 to 1.010	0.317
Diastolic blood pressure	0.979	0.945 to 1.014	0.243

eGFR indicates estimated glomerular filtration rate; gSEM, generalized structured equation modeling; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; NT-proBNP, N-terminal pro B-type natriuretic peptide; NYHA, New York Heart Association (functional class); TB4, thymosin beta-4.
*Statistically significant

median levels of NTproBNP and TB4. TB4 further split the risk group (P=0.027) in patients with below median levels of NT-proBNP (Figure S3). There were too few deaths in the subgroup of HFpEF (n=24) and HFrEF (n=36) to allow for further stratified analysis by HF type.

gSEM Modeling of TB4 and Clinical Outcomes

To evaluate whether TB4 has prognostic value independent of NT-proBNP in HF, we used gSEM to model TB4's contribution to all-cause mortality. The use of gSEM over conventional Cox regression modeling allowed us to account for potential interactions between variables. In the full model, NT-proBNP, age, NYHA class, and history of myocardial infarction were significant (Table 4). In determining the number of variables to retain, we followed the rule of 5 to 9 events per variable.[28] In our refined model (Table 5), we retained 4 variables: NT-proBNP, age, NYHA class, and history of myocardial infarction,

Table 5. gSEM Modeling for Time to All-Cause Mortality in Final Model

Time to Mortality	Adjusted Hazard Ratio	95% CI	*P*-Value
Analysis 1, all HF patients, n=415, 57 deaths			
Ln NT-proBNP	1.437	1.146 to 1.802	0.002*
Ln TB4	1.087	0.758 to 1.558	0.650
Age	1.041	1.016 to 1.067	0.001*
NYHA class			
2 vs 1	2.362	0.810 to 6.888	0.115
3&4 vs 1	7.593	2.496 to 23.096	<0.001*
Myocardial infarction	1.265	0.696 to 2.297	0.440
Analysis 2, men with HF, n=203, 30 deaths			
Ln NT-proBNP	1.459	1.041 to 2.047	0.028*
Ln TB4	0.791	0.426 to 1.467	0.456
Age	1.041	1.007 to 1.076	0.018*
NYHA class			
2 vs 1	2.244	0.663 to 7.598	0.194
3&4 vs 1	7.689	1.804 to 32.774	0.006*
Myocardial infarction	0.776	0.294 to 2.053	0.610
Analysis 3, Women with HF, n=212, 27 deaths			
Ln NT-proBNP	1.367	0.942 to 1.985	0.100
Ln TB4	1.668	1.033 to 2.691	0.036*
Age	1.044	0.998 to 1.092	0.064
NYHA class			
2 vs 1	3.350	0.416 to 26.972	0.256
3&4 vs 1	10.655	1.319 to 86.054	0.026*
Myocardial infarction	2.432	1.115 to 5.308	0.026*

gSEM, generalized structured equation modeling; HF, heart failure; NT-proBNP, N-terminal pro B-type natriuretic peptide; NYHA, New York Heart Association (functional class); TB4, thymosin beta-4.
*Statistically significant

which was significant in the full model (Table 4), and added TB4. In men, only age and NYHA class remained significant while in women, TB4 was independently predictive of 2-year all-cause mortality, alongside NYHA class and history of myocardial infarction. Likewise, we note the same trends were observed (including statistical significance of TB4) using the above inputs to a standard Cox regression model.

Discussion

Sex differences in HF are consistently seen in cohort studies.[1,2,29] Both the Framingham heart study[30] and the Rotterdam study[31] reveal that while lifetime risk of HF is similar in both sexes, clinical onset is earlier in men than women. Women also have a survival advantage over men upon diagnosis.[32–35] Ischemic heart disease dominates in men, while systolic hypertension dominates in women.[36] Potentially reflective of these risk factors, female patients are twice as likely as men to develop HFpEF.[3]

We studied endogenous plasma TB4 and found its level to be significantly elevated in HF. In a segmented analysis, we found that it was female HFpEF that accounted for the observed differences, although diagnostic performance of TB4 is still modest compared to NT-proBNP and hsTnT even in this subgroup. TB4 was a significant prognostic marker for 2-year mortality in female patients with HF via Kaplan–Meier analysis and gSEM modeling. We observed that inclusion of both NT-proBNP and hsTnT left a large proportion of gSEM variables insignificant. We thus chose NT-proBNP as the more stringent HF biomarker and omitted hsTnT from gSEM modeling. The relationship between TB4 and hsTnT may therefore benefit from additional study.

To gain insight into the functional context of TB4, we used a hierarchical clustering algorithm to define cross-correlative networks of soluble biomarkers in the 92-member multiplex panel. Applying a cutoff value of Pearson correlation coefficient (R) >0.7 and P-value $<10^{-10}$, we find that TB4 lies tightly within a cluster of 7 biomarkers, 6 of which are either X-linked or involved in sex hormone signaling. Thus, while we emphasize the need for replication studies, the nature of this correlative cluster is consistent with a sex differential role for TB4 in our studied cohort.

The top correlate was soluble plasma CD40 ligand, an X-linked immune mediator involved in inflammatory and vascular diseases,[37] which is regulated by NF-κB[38] and a marker of cardiac events in healthy women.[39] Soluble plasma CD40 ligand is elevated in diseases with strong female prevalence: Hashimoto thyroiditis (95% female),[40] systemic lupus erythematosus (88% female),[41] and Sjögren syndrome (92% female).[41] ITGB1BP2 (melusin) is also an X-linked gene and a known protective factor in cardiac injury.[42] Another correlate, NF-kappa-B essential modulator, is an X-linked gene that is cardioprotective in experimental HF through NF-κB-mediated modulation of oxidative stress.[43] HSP27 is also cardioprotective and activates the NF-κB pathway[44,45]; its secretion is regulated by estrogen[46] and shows elevated expression levels in HFpEF.[47] Epidermal growth factor and Src are both part of the extranuclear estrogen signaling network,[48] can also activate the NF-κB pathway,[49] and play a role in both vascular and immune processes.[50] The seventh member of the cluster, sirtuin-2, regulates NF-κB-dependent gene expression.[51]

Thus, within our analysis, TB4 is associated with markers of sex-specific biology as well as the NF-κB pathway, a central pathway in immune system activation, tolerance, and autoimmune pathophysiology.[52] TB4 has been shown to inhibit the NF-

κB pathway in a mouse model of myocardial infarction[53] and in tumor necrosis factor α–stimulated corneal epithelial cells.[54] We also note that inhibition of NF-κB resulted in suppression of TB4 expression in a prior study of melanoma cells.[55]

A mechanism for the higher levels of TB4 in females is suggested by the chromosomal location of the TB4 gene (TMSB4X). TMSB4X is located on the third evolutionary stratum of the X-chromosome (Xp22.2), a region highly predicted to XCi escape. The genes flanking TMSB4X on either side, TLR7/8 and FAM9C, each has empirical evidence for XCi escape.[56,57] We thus hypothesize that increased transcriptional dosing via XCi escape may contribute to sex differences in TB4.

Our current study is limited by cohort size, as the observed TB4 elevation is confined primarily to the female HFpEF subgroup. Likewise, the relatively small sample size, 106 male controls versus 221 male HF, may contribute to the statistical nonsignificance in males; thus, replication studies in additional cohorts will be required to fully establish TB4 sex specificity. Clinical outcomes were also limited because of sample and subsample sizes and a relatively short observation period of 2 years. However, given the body of evidence supporting TB4 as cardioprotective factor, our observations of TB4 levels in women with HF and multiple associations with sex-related biology, we hypothesize TB4 as a component of the immunopathophysiology of HF, potentially accentuated in women through increased transcriptional dosing and Xi escape. Next steps will include in vitro studies and prospective validation in both acute and compensated HF and acute cardiac injury.

Conclusions

We show for the first time that plasma TB4 is elevated in women with HFpEF, and predicts mortality independent of clinical risk factors and NT-proBNP in women with HF. Taken in context of known TB4 biology, our findings suggest that circulating TB4 reflects a compensatory response to cardiac injury, and may be a potential contributor to sex differences in HF.

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Disclosures

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SUPPLEMENTAL MATERIAL

Table S1. Baseline characteristics associated with baseline TB4, NT-proBNP or hsTnT from univariate linear regression in patients with heart failure

	TB4†		NT-proBNP†		hsTnT†	
	Coefficient	**95% CI (p-value)**	**Coefficient**	**95% CI (p-value)**	**Coefficient**	**95% CI (p-value)**
Clinical Characteristics						
Age, years	0.008*	0.001 to 0.015 (0.023)	0.031*	0.018 to 0.044 (<0.001)	0.015*	0.008 to 0.022 (<0.001)
Women vs. Men	0.358*	0.201 to 0.515 (<0.001)	-0.061	-0.359 to 0.236 (0.687)	-0.199*	-0.354 to -0.044
Race						
Malay vs. Chinese	-0.192*	-0.380 to -0.003 (0.046)	-0.083	-0.436 to 0.270 (0.645)	-0.012	-0.197 to 0.173 (0.900)
Indian vs. Chinese	0.317*	0.035 to 0.600 (0.028)	-0.512	-1.042 to 0.019 (0.059)	-0.201	-0.479 to 0.077 (0.156)
Body Mass Index, kg/m^2	0.014	-0.000 to 0.029 (0.054)	-0.080*	-0.106 to -0.055 (<0.001)	-0.023*	-0.037 to -0.009 (0.002)
Heart Rate, beats/min	0.002	-0.003 to 0.008 (0.412)	0.005	-0.006 to 0.016 (0.345)	-0.004	-0.010 to 0.002 (0.189)
Systolic BP, mmHg	-0.001	-0.004 to 0.003 (0.712)	-0.003	-0.010 to -0.004 (0.346)	-0.002	-0.006 to 0.001 (0.219)
Diastolic BP, mmHg	-0.011*	-0.018 to -0.004 (0.002)	-0.010	-0.023 to 0.003 (0.116)	-0.011*	-0.018 to -0.004 (0.001)
NYHA Class						
2 vs. 1	0.263*	0.067 to 0.459 (0.009)	0.530*	0.172 to 0.889 (0.004)	0.343*	0.150 to 0.536 (0.001)
3&4 vs. 1	0.435*	0.176 to 0.693 (0.001)	1.111*	0.638 to 1.583 (<0.001)	0.442*	0.188 to 0.697 (0.001)
Ischemic etiology of HF	-0.115	-0.275 to 0.046	0.385*	0.089 to 0.681	-0.259*	0.104 to 0.413

		(0.161)		(0.011)		(0.001)
Coronary Artery Disease	-0.110	-0.278 to 0.058 (0.200)	0.506*	0.206 to 0.805 (0.001)	0.266*	0.105 to 0.426 (0.001)
Hypertension	0.044	-0.154 to 0.242 (0.664)	-0.096	-0.459 to 0.266 (0.603)	0.231*	0.037 to 0.425 (0.020)
Diabetes Mellitus	0.064	-0.101 to 0.229 (0.444)	0.150	-0.154 to 0.454 (0.335)	0.384*	0.225 to 0.543 (<0.001)
Atrial Fibrillation	0.068	-0.116 to 0.251 (0.469)	0.536*	0.202 to 0.871 (0.002)	-0.090	-0.271 to 0.091 (0.328)
Peripheral Vascular Disease	0.799*	0.315 to 1.282 (0.001)	1.826*	0.741 to 2.911 (0.001)	0.657*	0.141 to 1.173 (0.013)
Cancer	0.036	-0.335 to 0.407 (0.848)	0.350	-0.484 to 1.183 (0.410)	0.133	-0.264 to 0.529 (0.511)
History of Smoking						
Ex-smoker vs. Non-smoker	-0.351*	-0.557 to -0.145 (0.001)	0.182	-0.200 to 0.564 (0.351)	0.086	-0.120 to 0.292 (0.412)
Current smoker vs. Non-smoker	-0.260*	-0.505 to 0.016 (0.037)	-0.568*	-1.021 to -0.116 (0.014)	-0.226	-0.469 to 0.018 (0.070)
Laboratory Values						
Sodium, mmol/L	0.001	-0.007 to 0.008 (-0.846)	0.006	-0.008 to 0.020 (0.429)	-0.002	-0.010 to 0.005 (0.532)
Haemoglobin, g/dL	-0.091	-0.132 to -0.051 (<0.001)	-0.198	-0.273 to -0.123 (<0.001)	-0.090	-0.130 to -0.050 (<0.001)
White blood cell, $10^3/\mu L$	0.037*	0.007 to 0.067 (0.016)	-0.069*	-0.124 to -0.013 (0.015)	-0.012	-0.042 to 0.018 (0.424)
Albumin, g/L	0.009*	0.004 to 0.014 (<0.001)	-0.198*	-0.273 to -0.123 (<0.001)	-0.090*	-0.130 to -0.050 (<0.001)
Creatinine, umol/L	0.001	-0.001 to 0.002 (0.291)	0.010*	-0.007 to 0.013 (<0.001)	0.007*	0.005 to 0.008 (<0.001)

eGFR, mL/min	-0.002	-0.005 to 0.001 (0.302)	-0.022*	-0.027 to -0.017 (<0.001)	-0.013*	-0.016 to -0.010 (<0.001)
TB4†, ng/L			0.088	-0.087 to 0.262 (0.324)	0.103*	0.012 to 0.195 (0.026)
NT-proBNP†, pg/mL	0.025	-0.025 to 0.076 (0.324)			0.299*	0.258 to 0.339 (<0.001)
hsTnT†, pg/mL	0.109*	0.013 to 0.205 (0.026)	1.081*	0.934 to 1.229 (<0.001)		
Medication						
Diuretic	0.150	-0.080 to 0.379 (0.200)	0.596*	0.173 to 1.019 (0.006)	0.215	-0.008 to 0.439 (0.059)
ACEI/ARB	-0.094	-0.261 to 0.074 (0.273)	-0.024	-0.335 to 0.288 (0.881)	-0.109	-0.273 to 0.054 (0.190)
Beta-blocker	-0.129	-0.346 to 0.088 (0.243)	0.244	-0.160 to 0.647 (0.236)	-0.046	-0.258 to 0.166 (0.673)
Aldosterone antagonist	-0.280*	-0.456 to -0.103 (0.002)	0.201	-0.129 to 0.532 (0.232)	-0.051	-0.225 to 0.123 (0.564)
Digoxin	0.108	-0.087 to 0.303 (0.277)	0.572*	0.213 to 0.930 (0.002)	-0.068	-0.259 to 0.122 (0.481)
Statin	0.191	-0.028 to 0.411 (0.087)	-0.047	-0.455 to 0.362 (0.822)	-0.037	-0.251 to 0.178 (0.738)
Echocardiographic Data						
LVEF in %	0.010*	0.005 to 0.015 (<0.001)	-0.039*	-0.047 to -0.031 (<0.001)	-0.010*	-0.014 to -0.005 (<0.001)
Mitral E/e' Ratio	-0.004	-0.012 to 0.004 (0.310)	0.057*	0.044 to 0.070 (<0.001)	0.020*	0.013 to 0.027 (<0.001)

*** Variables significantly associated with baseline TB4, NT-proBNP or hsTnT with p-value < 0.05**

†Natural logarithm of TB4, NT-proBNP and hsTnT.

Table S2. Independent Correlates of NT-proBNP From Univariate Analysis Followed by Multivariate Analysis With Backward Elimination.

Ln NT-proBNP, n=320, adjusted R^2=0.555			
Clinical Variables	**Coefficient**	**95% CI**	**p-value**
Body Mass Index, kg/m^2	-0.041	-0.062 to -0.020	<0.001
Atrial Fibrillation	0.772	0.519 to 1.025	<0.001
eGFR, mL/min	-0.012	-0.016 to 0.007	<0.001
hsTnT*, pg/mL	0.724	0.564 to 0.884	<0.001
LVEF in %	-0.024	-0.031 to -0.017	<0.001
Mitral E/e' Ratio	0.025	0.014 to 0.035	<0.001

*Natural logarithm of hsTnT
eGFR = estimate glomerular filtration rate; LVEF = Left ventricle ejection fraction

Table S3. Independent Correlates of hsTnT From Univariate Analysis Followed by Multivariate Analysis With Backward Elimination.

Ln hsTnT, n=350, adjusted R^2=0.445			
Clinical Variables	**Coefficient**	**95% CI**	**p-value**
Women vs. Men	-0.196	-0.326 to -0.066	0.003
NYHA Class			
2 vs. 1	0.227	0.070 to 0.384	0.005
3&4 vs. 1	0.100	-0.116 to 0.315	0.364
Diabetes Mellitus	0.229	0.096 to 0.362	0.001
Albumin, g/L	0.004	0.000 to 0.008	0.047
eGFR, mL/min	-0.007	-0.010 to -0.004	<0.001
NT-proBNP*, pg/mL	0.247	0.201 to 0.294	<0.001
TB4#, ng/ml	0.095	0.020 to 0.171	0.014

*Natural logarithm of NT-proBNP and TB4
NYHA = New York Heart Association (functional class); eGFR = estimate glomerular filtration rate

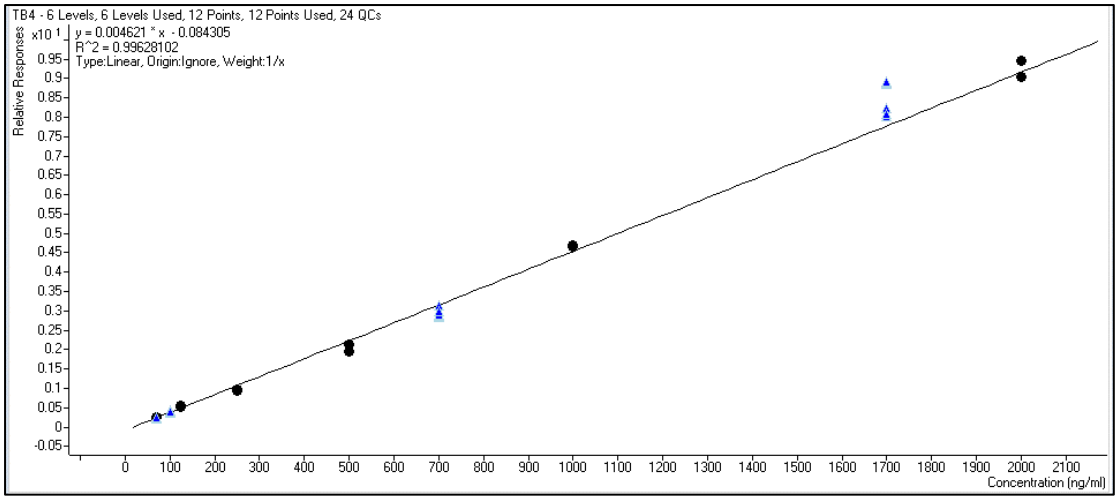

Figure S1. **Qualification batch for TB4 in the SHOP cohort studies. Each point is based on TB4 MRM transition (m/z) 709.3 > 810 normalized over the internal standard of TB4 with MRM transition (m/z) 713> 658.4. The calibration curve consists of 6 standard concentration levels of 70, 125, 250, 500, 1000 and 2000ng/mL in duplicates (•), and total 6 QC samples (▲) divided on three different levels of 100ng/mL (low), 700ng/mL (medium) and 1700ng/mL (high)). The lower limit of quantification (LLOQ) is 70ng/mL and upper limit of quantification (ULOQ) is 2000ng/mL. All standard and QCs concentrations for all qualification and analytical runs were ≤ ±15% (≤ ±15% at LLOQ) within the nominal value, with % CV ≤ 5.**

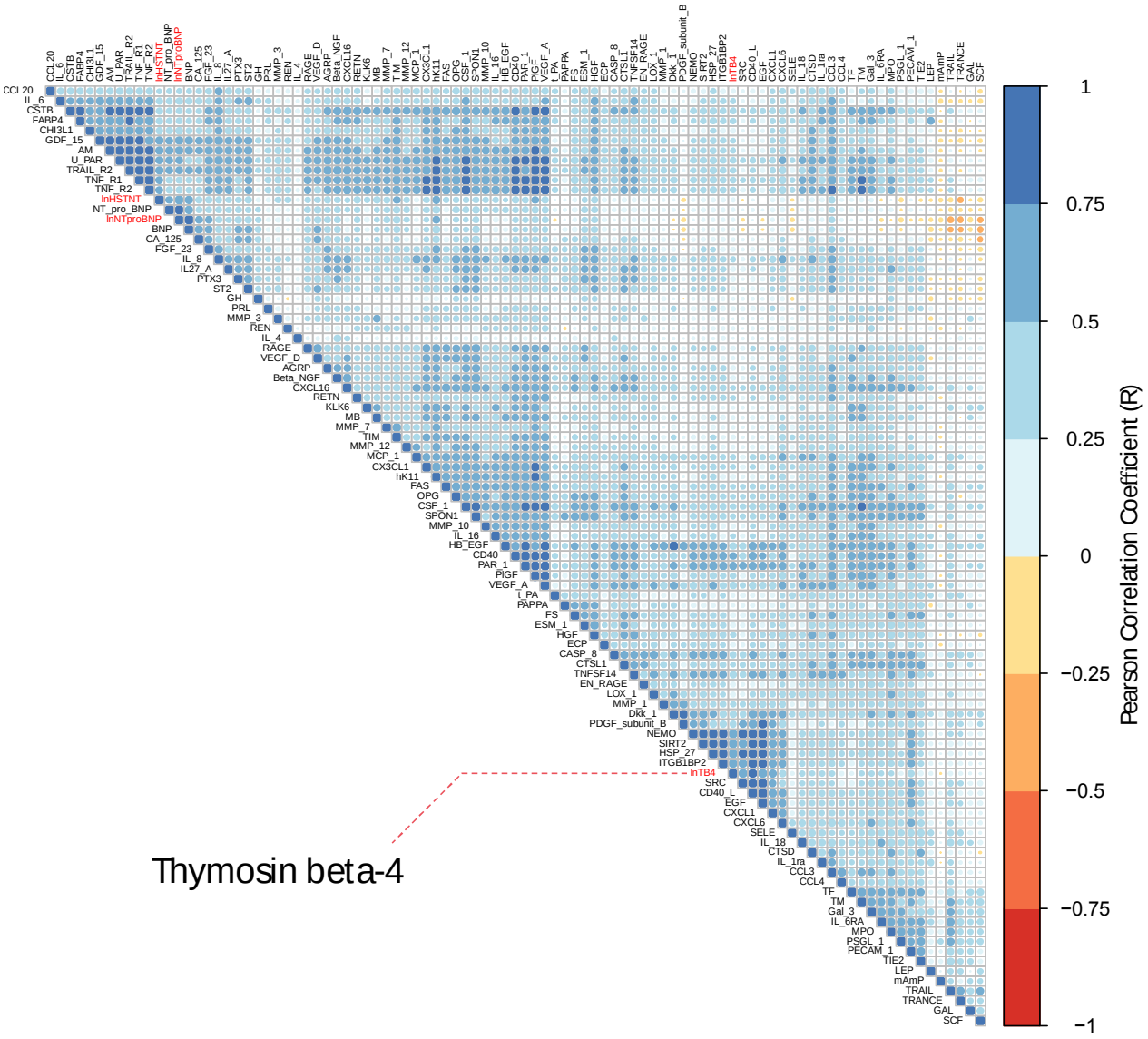

Figure S2. Correlation matrix of biomarkers measured in the full cohort (n=657), ordered by hierarchical clustering. Size and color of circles represent R values. Biomarkers in red were measured independently of the Proseek panel. Full name of proteins and uniprot accession number may be obtained from Table S4.

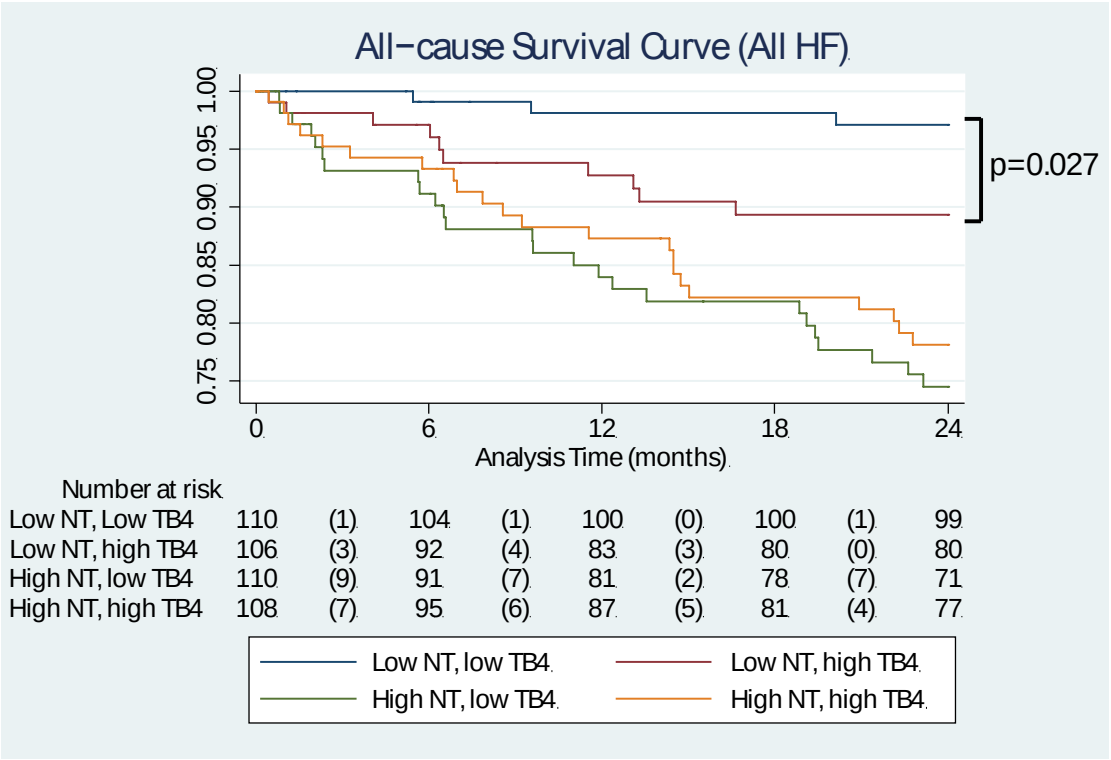

Figure S3. HF Patients were split into four groups based on whether they were above or below median in levels of NT-proBNP and TB4.