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SHORT COMMUNICATION

Sex-specific cardiovascular protein levels and their link with clinical outcome in heart failure

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Abstract

Aims This study aims to provide insight into sex-specific cardiovascular protein profiles and their associations with adverse outcomes, which may contribute to a better understanding of heart failure (HF) pathophysiology and the optimal use of circulating proteins for prognostication in women and men.

Methods and results In 250 stable patients with HF with reduced ejection fraction (HFrEF), we performed trimonthly blood sampling (median follow-up: 26 [17–30] months). We selected all baseline samples and two samples closest to the primary endpoint (PEP; composite of cardiovascular death, heart transplantation, left ventricular assist device implantation, and HF hospitalization) or one sample closest to censoring and applied the Olink Cardiovascular III panel. We used linear regression to study sex-based differences in baseline levels and joint models to study differences in the prognostic value of serially measured proteins. In 66 women and 184 men (mean age of 66 and 67 years, respectively), 21% and 28% reached the PEP, respectively. Mean baseline levels of fatty acid-binding protein 4, secretoglobin family 3A member 2, paraoxonase 3, and trefoil factor 3 were higher in women ($P_{interaction}$: 0.001, 0.007, 0.018, and 0.049, respectively), while matrix metalloproteinase-3, interleukin 1 receptor-like 1, and myoglobin were higher in men ($P_{interaction}$: <0.001, 0.001, and 0.049, respectively), independent of clinical characteristics. No significant differences between sexes were observed in the longitudinal associations of proteins with the PEP. Only peptidoglycan recognition protein 1 showed a suggestive interaction with sex for the primary outcome ($P_{interaction}$ = 0.028), without multiple testing correction, and was more strongly associated with adverse outcome in women {hazard ratio [HR] 3.03 [95% confidence interval (CI), 1.42 to 6.68], *P* = 0.008} compared with men [HR 1.18 (95% CI, 0.84 to 1.66), *P* = 0.347].

Conclusions Although multiple cardiovascular-related proteins show sex differences at baseline, temporal associations with the adverse outcome do not differ between women and men with HFrEF.

Keywords Sex differences; Proteomics; Heart failure; HFrEF

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Introduction

While sex-specific differences have been observed in incidence, relative impact of risk factors, and treatment response in heart failure (HF), including HF with reduced ejection fraction (HFrEF), the exact mechanisms underlying these differences remain poorly understood.[1,2] Insight into sex-specific cardiovascular protein profiles and their associations with the risk of adverse outcomes may contribute to a better understanding of the pathophysiological processes involved in HFrEF and guide the optimal use of circulating proteins in cardiovascular risk estimation systems in women and men. Previous studies focusing on sex differences in circulating proteins in patients with stable HFrEF are scarce and generally focused on a limited number of proteins and on single baseline measurements.[3,4] Distinguishing patients at different levels of

risk of adverse events based on a single protein measurement is challenging, and serial protein measurements may contribute to dynamic risk assessment.

In the current investigation, we have serially measured 92 circulating proteins, previously associated with cardiovascular disease, in 66 women and 184 men with stable HFrEF. We examined sex-based differences in protein profiles and the association of serially measured proteins with adverse cardiovascular events in women and men.

Methods

Study population

The *Serial Biomarker Measurements and New Echocardiographic Techniques in Chronic Heart Failure Patients Result in Tailored Prediction of Prognosis* (Bio-SHiFT) study is a prospective cohort study of stable patients with chronic HF, conducted in Erasmus MC, Rotterdam, and Northwest Clinics, Alkmaar, Netherlands. The study design has been described in more detail previously.[5] In brief, Bio-SHiFT enrolled stable outpatients ≥18 years old with HFrEF. Study follow-up visits and blood sample collection were scheduled every 3 months. The primary endpoint (PEP) comprised the composite of cardiovascular death, heart transplantation, left ventricular assist device implantation, and hospitalization for the management of acute or worsened HF (Supporting Information, *Table S1*). The medical ethics committee of the Erasmus Medical Center in Rotterdam approved the study protocol, and all patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and registered in ClinicalTrial.gov (NCT01851538). For the current investigation, the first inclusion round was used, during which a total of 250 patients were enrolled.

Sample selection and proteomic analysis

For the current investigation, all baseline blood samples were selected. Additionally, the last two samples drawn before the PEP were selected, or the last sample that was available before censoring in patients who remained endpoint free. The time interval between moment of sampling and the PEP or censoring was similar for women and men (Supporting Information, *Table S2*). The Olink Multiplex platform (Olink Proteomics AB, Uppsala, Sweden) Cardiovascular III panel was used to measure 92 plasma proteins.[6,7] The readout of the Olink assay is in normalized protein expression (NPX) and is expressed on a log2 scale. Accordingly, one unit higher NPX value represents a doubling of the measured protein concentration. In total, data from 533 samples passed the quality-control criteria.

Statistical analysis

Linear regression using only baseline measurements was applied to reveal sex-based differences in mean protein baseline levels while accounting for potential differences in clinical features and comorbidities by adjusting for the MAGGIC HF mortality risk score.[8] Sex-based differences in the prognostic value of serially measured circulating protein concentrations were evaluated using joint models for each individual protein, including the MAGGIC score and an interaction term for sex and protein level. Joint modelling combines a linear mixed-effects model to describe the trajectory of a predictor with a time-to-event relative risk model to relate the estimated temporal pattern of a predictor with the hazard of the outcome of interest (*Supplemental Methods*).[9] Given the limited number of patients (specifically, four individuals) who experienced non-cardiovascular deaths without prior occurrence of the other components of the PEP, we did not perform competing risk analysis. In a secondary analysis, sex-based differences in the prognostic value of serially measured protein concentrations were evaluated using joint models, but with adjustments for age and body mass index (BMI) only.

Data analyses were performed using R (Version 4.1.2.). Missing values of clinical characteristics were imputed using single imputation. *P*-values were corrected for multiple testing using the Benjamini–Hochberg method (false discovery rate < 0.05).

Results

Clinical characteristics of the study population

The total study population comprised 66 (26.4%) women and 184 (72.8%) men with a similar mean age (66 ± 13 vs. 67 ± 13, $P = 0.607$) (*Table 1*). At baseline, women had a significantly lower mean BMI (26.1 ± 4.5 vs. 28.0 ± 4.6, $P = 0.004$) and were more often current smokers (16.7% vs. 8.7%, $P = 0.002$) than men. The aetiology of HF was less often ischaemic in women (25.8% vs. 53.8%, $P < 0.001$). Moreover, the prevalence of comorbidities, such as a history of myocardial infarction (21.5% vs. 44.2%, $P = 0.002$) and known hypercholesterolaemia (27.3% vs. 43.8%, $P = 0.029$), was also significantly lower in women. No differences in mean left ventricular ejection fraction (women: 30 ± 10 vs. men: 30 ± 10, $P = 0.913$) were present between sexes.

Sex-based differences in cardiovascular protein concentrations at baseline

After correction for multiple testing, significant differences between women and men were observed in mean baseline

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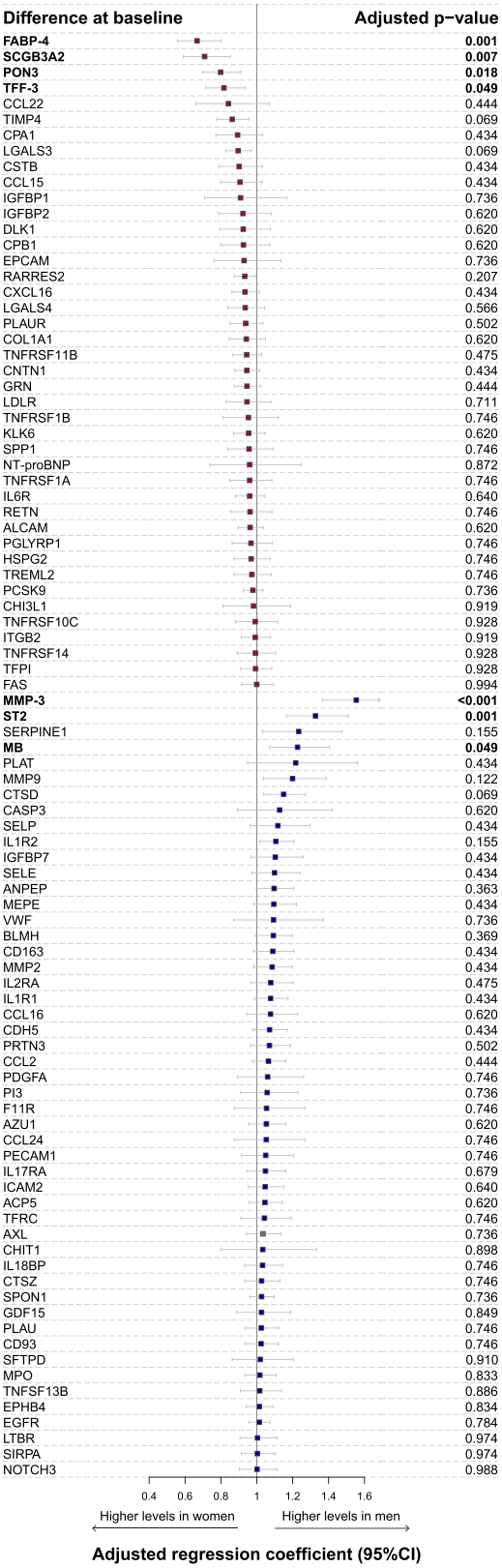
Table 1 Baseline characteristics of study population

	Total population $n = 250$	Women $n = 66$	Men $n = 184$	*P*-value
Demographics				
Age, years, mean (SD)	66.3 (12.7)	65.6 (13.4)	66.5 (12.5)	0.607
Caucasian ethnicity (%)	231 (93.5)	61 (95.3)	170 (92.9)	0.703
Clinical characteristics				
Body mass index, kg/m^2, mean (SD)	27.5 (4.7)	26.1 (4.5)	28.0 (4.6)	**0.004**
Systolic blood pressure, mmHg, mean (SD)	121.5 (20.5)	122.0 (22.0)	121.4 (20.1)	0.835
Diastolic blood pressure, mmHg, mean (SD)	72.2 (10.9)	72.5 (11.2)	72.1 (10.8)	0.808
Features of heart failure				
Duration of HF, years[a]	4.8 [1.8, 9.7]	3.7 [1.3, 7.3]	5.1 [2.2, 10.2]	0.087
NYHA class (%)				0.965
NYHA class I and II	188 (75.2)	49 (74.2)	139 (75.5)	
NYHA class III and IV	62 (24.8)	17 (25.8)	45 (24.5)	
LVEF, mean (SD)[b]	29.8 (9.8)	29.9 (10.2)	29.7 (9.6)	0.913
Heart failure aetiology				
Ischaemic heart disease (% yes)	116 (46.4)	17 (25.8)	99 (53.8)	**<0.001**
Cardiomyopathy (% yes)	63 (25.2)	19 (28.8)	44 (23.9)	0.537
Hypertension (% yes)	31 (12.4)	13 (19.7)	18 (9.8)	0.060
Secondary to valvular heart disease (% yes)	10 (4.0)	3 (4.5)	7 (3.8)	0.726
Other aetiology (% yes)	12 (4.8)	5 (7.6)	7 (3.8)	0.371
Unknown aetiology (% yes)	18 (7.2)	9 (13.6)	9 (4.9)	**0.037**
Established biomarker levels				
NT-proBNP (pmol/L)[a]	133.2 [44.9, 274.4]	108.8 [49.4, 250.4]	138.4 [44.9, 283.9]	0.632
Hs-TnT (ng/L)[a]	17.7 [9.4, 32.7]	11.7 [7.3, 26.1]	19.5 [10.7, 36.6]	**0.002**
CRP (mg/L)[a]	2.2 [0.9, 4.9]	2.2 [0.9, 4.7]	2.1 [0.9, 4.9]	0.775
Medical history				
Myocardial infarction (% yes)	94 (38.2)	14 (21.5)	80 (44.2)	**0.002**
PCI (% yes)	81 (32.4)	14 (21.2)	67 (36.4)	**0.035**
CABG (% yes)	42 (16.8)	5 (7.6)	37 (20.1)	**0.032**
Atrial fibrillation (% yes)	97 (39.6)	19 (29.2)	78 (43.3)	0.065
CRT (% yes)	78 (31.2)	19 (28.8)	59 (32.1)	0.735
Pacemaker (% yes)	41 (17.3)	10 (15.9)	31 (17.8)	0.877
Chronic renal failure (% yes)	131 (52.8)	37 (56.1)	94 (51.6)	0.637
Diabetes mellitus (% yes)	77 (30.8)	19 (28.8)	58 (31.5)	0.797
Hypercholesterolaemia (% yes)	95 (39.3)	18 (27.3)	77 (43.8)	**0.029**
COPD (% yes)	32 (12.9)	7 (10.8)	25 (13.7)	0.702
Intoxications				
Smoking (%)				**0.002**
Never	68 (27.3)	26 (39.4)	42 (23.0)	
Current	27 (10.8)	11 (16.7)	16 (8.7)	
Former (>30 days)	154 (61.8)	29 (43.9)	125 (68.3)	
Medication use				
Beta-blockers (% yes)	225 (90.0)	58 (87.9)	167 (90.8)	0.667
ACE-I (% yes)	169 (67.6)	43 (65.2)	126 (68.5)	0.732
ARB (% yes)	72 (28.8)	22 (33.3)	50 (27.2)	0.430
Aldosterone antagonist (% yes)	174 (69.6)	47 (71.2)	127 (69.0)	0.860
Loop diuretics (% yes)	226 (90.4)	62 (93.9)	164 (89.1)	0.371
Thiazide diuretics (% yes)	6 (2.4)	0 (0.0)	6 (3.3)	0.345
Aspirin (% yes)	45 (18.1)	13 (19.7)	32 (17.5)	0.831
Anticoagulants (% yes)	193 (77.2)	46 (69.7)	147 (79.9)	0.128
MAGGIC risk score				
MAGGIC risk score, mean (SD)	20.7 (7.3)	19.6 (8.1)	21.1 (6.9)	0.151

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CABG, coronary artery bypass graft; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CRT, cardiac resynchronization therapy; hs-TnT, high-sensitivity troponin T; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-hormone B-type natriuretic peptide; NYHA, New York Heart Association; PCI, percutaneous coronary intervention; SD, standard deviation.
Bold indicates a *P*-value < 0.05 that is considered statistically significant.
[a]All biomarker levels and duration of heart failure (HF) are presented as median [25th–75th percentile].
[b]Missing for 51 patients.

concentration for seven proteins (*Figure 1*). Specifically, fatty acid-binding protein 4 (FABP-4), secretoglobin family 3A member 2 (SCGB3A2), paraoxonase 3 (PON3), and trefoil factor 3 (TFF-3) showed higher mean levels in women. Conversely, mean levels of matrix metalloproteinase-3 (MMP-3), interleukin 1 receptor-like 1 [suppression of tumorigenesis-2 (ST2)], and myoglobin (MB) were higher in men. These seven proteins were mostly associated with age, diabetes mellitus,

Figure 1 Sex-based differences in protein levels at baseline. The mean difference (i.e. the regression coefficient for sex) between women and men at baseline is depicted for all 92 circulating proteins. Proteins showing statistically significant differences (false discovery rate < 0.05) between women and men are depicted in bold. Analyses were adjusted for the MAGGIC risk score.



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and N-terminal pro-hormone B-type natriuretic peptide concentration in the current study (Supporting Information, *Table S3*). Moreover, a similar pattern in the sex-specific concentrations of these proteins was found in patients with and without the PEP (Supporting Information, *Figure S1*) and during follow-up (Supporting Information, *Figure S2*). Interestingly, during follow-up, five additional proteins showed sex-related differences: Galectin 3 (Gal-3; LGALS3), retinoic acid receptor responder 2 (RARRES2), CXC chemokine ligand 16 (CXCL16), contactin 1 (CNTN1), and granulin (GRN) exhibited significantly higher levels in women compared with men.

Sex-based differences in the prognostic value of serially measured circulating proteins

During a median [25th–75th percentile] follow-up of 26 [17–30] months, a total of 14 female and 52 male patients reached the PEP (Supporting Information, *Table S1*).

No significant differences were observed in the longitudinal associations of circulating proteins with an adverse cardiovascular outcome between women and men after adjustment for multiple testing (Supporting Information, *Table S4*). Only peptidoglycan recognition protein 1 (PGLYRP1) showed a suggestive interaction with sex for the PEP ($P_{interaction}$ = 0.028), without multiple testing correction, and was more strongly associated with adverse outcome in women {hazard ratio [HR] 3.03 [95% confidence interval (CI), 1.42 to 6.68], P = 0.008} compared with men [HR 1.18 (95% CI, 0.84 to 1.66), P = 0.347]. Subsequent secondary analyses, with model adjustments solely for age and BMI, did not reveal any statistically significant interactions with sex for the proteins included in our study (Supporting Information, *Table S5*). Secreted phosphoprotein 1 (SPP1), also known as osteopontin, demonstrated a suggestive interaction with sex regarding the PEP ($P_{interaction}$ = 0.040).

Discussion

We examined levels of 92 cardiovascular-related proteins in women and men with HFrEF and their associations with clinical outcomes. We found sex differences in baseline levels of several circulating proteins previously associated with cardiovascular disease. However, no significant differences were observed between women and men in the longitudinal associations of circulating proteins with an adverse cardiovascular outcome.

Previous studies on sex differences in circulating proteins in HF are limited in number but have already suggested sex differences in the absolute concentrations of established biomarkers.[10,11] We extend current knowledge by evaluating an elaborate set of 92 plasma proteins to identify sex-based differences in protein levels and in associated cardiovascular-related pathophysiological processes within various organ systems. We observed that circulating proteins associated with lipid metabolism (FABP-4),[12] cell/tissue repair (TFF-3),[13] and oxidative stress (PON3)[6] were overrepresented in women while circulating proteins reflecting cardiac remodelling (MMP-3 and ST2)[14,15] and cardiac injury (MB)[16] were overrepresented in men. SCGB3A2, overrepresented in women, is a newly discovered biomarker with prominent anti-inflammatory and anti-fibrotic activity in animal models of pulmonary fibrosis.[17] Data on the (sex-specific) prognostic role of SCGB3A2 in HF are currently lacking. Previous studies on sex differences in protein levels in patients with HF with preserved ejection fraction (HFpEF), a condition more prevalent in women, have also revealed significant sex-related disparities. For instance, Stienen *et al.*[18] identified nine proteins associated with among others lipid metabolism, haemostasis, and extracellular matrix organization, showing differential expression between female and male patients with chronic HFpEF. It should be kept in mind that baseline sex-related differences do not necessarily indicate sex-specific pathophysiological processes but could also result from inherent physiological differences between women and men. The role of sex hormones in this context should be further elucidated in (longitudinal) studies that also enrol women in the years prior to the menopause.

So far, most studies on the sex-specific associations of circulating proteins with clinical outcome of HF examined limited numbers (one or a few) of biomarkers, usually performed cross-sectional measurements only, and related them to adverse events occurring over many years thereafter.[3,4,18,19] Our highly frequent blood sampling design enabled us to account for the temporal changes and dynamic nature of HF and to study the association of repeatedly measured circulating proteins with clinical outcomes during follow-up. Out of the 92 repeatedly measured proteins, only PGLYRP1, previously associated with coronary artery disease and HF,[20] showed a sex-specific association with the PEP. Our findings are in line with recent studies by Raafs *et al.*[19] and Suthahar *et al.*,[3] which reported no statistically significant differences in the associations of 252 and 10 circulating proteins, respectively, with incident HF between women and men. In our previous investigations of sex differences in patients with chronic HF, we applied an aptamer-based multiplex proteomic approach and demonstrated that only 2 out of 1105 circulating proteins showed differing associations with adverse cardiovascular outcomes between women and men.[21] We now extend these previous findings by assessing sex differences using a multiplexed immune-based assay, thereby further exploring the proteomic landscape to gain a more comprehensive understanding of the underlying mechanisms. Differences between our prior findings and the findings of the current study can largely be explained by differences in

proteomic panel composition. Five [FABP-4, secretoglobin family 2A member 2 (SCGB2A2), PON3, TFF-3, and MMP-3] out of the seven proteins showing sex-related differences in the current study were not present in the aptamer-based panel. Conversely, only 6 [cathepsin D (CTSD), ST2, Gal-3, MB, tissue inhibitor of matrix metalloproteinase-4 (TIMP-4), and osteoprotegerin (OPG)] out of the 55 proteins identified in our previous investigations were present on the Olink panel. Of these six, two (ST2 and MB) were also identified in the current investigation. The direction of effect for the four remaining proteins was similar in both studies, but statistical significance was not retained after correcting for multiple testing.

The differences that we found in the protein profiles may be relevant for clinical practice, even if no sex differences are present in the associations of circulating proteins with clinical outcomes of HF. This is because, in spite of previous studies having demonstrated that women with HF are at lower risk of adverse events than men, uniform protein-level thresholds are usually applied to assess risk. If a uniform protein threshold is applied, women with elevated protein levels above the threshold will still have a lower absolute risk of adverse events than their male counterparts, which indicates that a sex-specific interpretation is warranted when using such an approach to circulating proteins for risk stratification.

Some limitations need to be acknowledged. First, the Bio-SHiFT study was not designed to examine differences between women and men; therefore, statistical power for this investigation was limited, particularly in women (27% of the study population). Further to this, the current investigation was limited to the first inclusion round (August 2011 to June 2013) of the Bio-SHiFT study, which enrolled patients up to January 2018. The observed sex-related differences, while interesting, should be considered as initial observations that require validation in larger, more focused studies that also explore associations with the separate components of our composite endpoint. Finally, the Bio-SHiFT study comprises a mostly White population, and generalizing our findings to other ethnic groups should be performed with caution.

Conclusions

Although multiple cardiovascular-related proteins show different levels between women and men at baseline, the association between these 92 proteins and adverse outcome does not significantly differ between women and men with chronic HFrEF.

Conflict of interest

J.J.B. received an independent research grant for ISS from Abbott to the hospital. J.J.B. has had speaker engagements and advisory boards in the past 5 years with Abbott, AstraZeneca, Bayer, Boehringer Ingelheim, Daiichi Sankyo, Novartis, and Vifor (all outside the submitted work). The other authors report no relationships that could be construed as a conflict of interest.

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Supporting information

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

Figure S1. Sex-specific differences in protein levels between patients with and without the primary endpoint. The mean difference (i.e., the regression coefficient for PEP) between patients with and without the primary endpoint (PEP) at baseline is depicted for all patients and for women and men separately. None of the proteins showed statistically significant differences (*P*-value <0.05) between women and men in their association with the PEP. Analyses were adjusted for the MAGGIC risk score.

Figure S2. Sex-based differences in protein levels during follow-up. The mean difference (i.e., the regression coefficient for sex) between women and men during follow-up is depicted for all 92 circulating proteins. Proteins showing statistically significant differences (FDR < 0.05) between women and men are depicted in bold. Analyses were adjusted for the MAGGIC risk score. This analysis incorporates all available samples per patient, including those collected during follow-up (n = 533).

Table S1. Follow-up and study endpoints.

Table S2. Time interval between moment of sampling and the PEP or censoring.

Table S3. Association between baseline characteristics and circulating proteins.

Table S4. Sex-specific associations with the primary endpoint, adjusted for the MAGGIC risk score.

Table S5. Sex-specific associations with the primary endpoint, adjusted for age and BMI.

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