

Pro-inflammatory biomarkers in women with non-obstructive angina pectoris and coronary microvascular dysfunction

Jakob Schroder^{a,*}, Naja Dam Mygind^{a,b}, Daria Frestad^a, Marie Michelsen^a, Hannah Elena Suhrs^a, Kira Bang Bove^a, Ida Gustafsson^a, Jens Kastrup^b, Eva Prescott^a

^a Department of Cardiology, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark

^b Department of Cardiology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

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ABSTRACT

Background: Studies that evaluate larger numbers of protein biomarkers in patients with coronary microvascular dysfunction (CMD) have not previously been performed, and very little is known concerning the pathogenetic mechanisms leading to CMD.

Our objective was to analyze associations between a broad cardiovascular disease (CVD) protein biomarker assay and CMD, and further explore internal biomarker relations in order to identify possible targets for future treatment interventions.

Methods: In 174 women with angina pectoris and no significant obstructive coronary artery disease (<50% stenosis on invasive coronary angiography), CMD was assessed by transthoracic Doppler echocardiography measuring coronary flow velocity reserve (CFVR). Blood samples were analyzed with a CVD proteomic panel encompassing 92 biomarkers. The relation between biomarkers and CFVR was evaluated by regression analysis, and possible interrelations between significant biomarkers were investigated by principal component analysis (PCA).

Results: Median age (SD) was 64 years (9.8), median CFVR (IQR) was 2.3 (1.9–2.7), and 28% of patients had CFVR < 2.0. Eighteen biomarkers were significantly correlated with CFVR. In PCA, 8 of the biomarkers significantly related to CFVR showed high loadings on principal component 1 (PC1). The component scores of PC1 were significantly related to CFVR ($p = 0.002$). The majority of the 8 interrelated PC1 biomarkers were related to the pro-inflammatory TNF- α – IL-6 – CRP pathway.

Conclusion: Eighteen protein biomarkers were significantly associated with CMD. Eight biomarkers were interrelated in PCA, and share connection with pro-inflammatory pathways, highlighting a possible important role of inflammation in CMD.

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1. Introduction

Coronary microvascular dysfunction (CMD) is the possible cause of symptoms in a substantial proportion of patients with angina and no obstructive coronary artery disease (CAD) [1]. Development of novel simple diagnostic tests and effective treatment in this large patient group is hindered by an incomplete understanding of the pathogenetic process leading to CMD. Compared with macrovascular cardiovascular disease (CVD), protein biomarker studies in CMD have been scarce and a recent CMD White Paper called for more studies of functionally related proteins, which may guide targeted intervention in CMD [2].

We have recently identified 4 protein biomarkers associated with CMD evaluated by rubidium-82 positron emission tomography (PET) in 97 patients [3]. In the present study we analyzed a range of CVD biomarkers with the aim of identifying common internal patterns representing pathophysiologic pathways which may be activated in patients with CMD.

2. Patients and methods

2.1. Enrollment, inclusion, baseline data

Participants with angina-like symptoms and no significant obstructive coronary artery disease (<50% coronary artery stenosis) were enrolled between March 2012 and September 2014, according to the inclusion criteria for the iPOWER study [4] (see Supplemental materials).

* Corresponding author.

E-mail address: jakob.arnborg.schroeder.02@regionh.dk (J. Schroder).

Abbreviations, alphabetical order

ACS	acute coronary syndrome
BMI	body mass index
CAD	coronary artery disease
CFVR	coronary flow velocity reserve
CMD	coronary microvascular dysfunction
CVD	cardiovascular disease
LoD	limit of detection
MI	myocardial infarction
PC	principal component
PCA	principal component analysis
PET	positron emission tomography

Biomarkers

CCL2	C-C motif ligand 2
CCL15	C-C motif ligand 15
CCL16	C-C motif ligand 16
CHI3L1	Chitinase-3-like protein 1
CXCL16	C-X-C motif ligand 16
Gal4	Galectin-4
GDF15	growth differentiation factor 15
MMP-3	matrix metalloproteinase 3
MMP-9	matrix metalloproteinase 9
NTproBNP	N-terminal prohormone of brain natriuretic peptide
PGLYRP1	peptidoglycan recognition protein 1
PON3	paraoxonase 3
SCGB3A2	secretoglobulin family 3a member 2
suPAR	soluble urokinase-type plasminogen activator receptor
TNF- α	tumor necrosis factor α
TNFR1	tumor necrosis factor receptor 1
tPA	tissue plasminogen activator
TRAIL	TNF-related apoptosis-inducing ligand

A subgroup of the iPOWER cohort was randomly selected for protein biomarker evaluation in this study.

2.2. Echocardiographic examination and coronary flow velocity reserve measurements

All patients underwent coronary flow velocity reserve (CFVR) measurement by transthoracic Doppler echocardiography of the left anterior descending artery at rest and during maximum hyperemia induced by dipyridamole infusion (0.84 mg/kg over 6 min) [5]. Left ventricular ejection fraction was analyzed by an experienced echocardiographer using the GE EchoPac v. 112 automated biplane calculation Auto-EF tool. For details regarding echocardiographic examination specifics, please see the Supplemental materials.

2.3. Protein biomarkers

All blood samples were analyzed with the Olink cardiovascular disease panel III (CVD III, Olink Proteomics, Uppsala, Sweden) using Proximity Extension Assay technology, measuring 92 protein biomarkers related to cardiovascular or other diseases by real-time polymerase chain reaction (See Online Fig. 1 for biomarker list. See Supplemental materials for a detailed description of the protein biomarker analysis process) [6].

2.4. Statistical analyses

Baseline patient characteristics were analyzed for possible associations with CFVR both as a continuous variable using linear regression and

dichotomized (<2.5 and ≥ 2.5) using *t*-tests and χ^2 -tests for continuous and categorical baseline variables, respectively.

The relation between each of the 92 protein biomarkers and the primary outcome, CFVR, was assessed using linear regression analysis after plotting each biomarker against CFVR for possible non-linear associations. Model assumptions were assessed graphically for all biomarkers and were best satisfied with log-2 transformed biomarker values (covariate) and log-2 transformed CFVR values (outcome) [3]. Family-wise error correction procedures (e.g. Bonferroni) or false discovery rate correction were not possible to utilize due to the large number of protein biomarkers and relatively small patient cohort ($n = 174$).

In an attempt to explore internal patterns within the group of protein biomarkers significantly associated with CFVR, a scaled principal component analysis (PCA) was performed. Details of the procedure are described in the Supplemental materials. Briefly, PCA is a statistical tool for data exploration and simplification which enables reduction of a larger number of variables into a smaller number of summary components, while still retaining as much of the variance from the original variables as possible [7]. In recent years PCA has been employed in various fields of health research, e.g. for data exploration purposes and development of clinical risk scores [8,9]. The PCA procedure may be subdivided into 3 steps [10,11]:

- Component extraction and selection: Significant protein biomarker internal correlations were examined by creating a Pearson correlation matrix followed by calculation of Kaiser-Meyer-Olkin measures and Bartlett's test statistic for sphericity. The cut-off eigenvalue level was set conservatively at 1.2 to avoid false discoveries. To simplify component interpretation, no component rotation was employed.
- Interpretation of component loading structure: We determined a threshold for component/variable loading >0.25 as meaningful for interpretation i.e. a specific variable was considered to be associated with a specific component if the loading was above this threshold [12].
- Computation and utilization of component scores: The loading coefficients were utilized to compute a separate component score for each patient and each extracted component. The component scores were then used to perform both univariate and multivariate adjusted regression analyses for association between components and CFVR, adjusting for the known risk factors for low CFVR which were found to be significantly associated with CFVR in the present study: Age, non-obstructive atherosclerosis in CAG, hypertension, diabetes mellitus, smoking, body mass index (BMI) and total cholesterol [2].

To obtain a preliminary overview of the internal relations between all protein biomarkers, an initial PCA including all 92 biomarkers was also performed; however, due to the high number of protein biomarkers relative to the cohort size the results were uninterpretable and did not fulfill methodological requirements of PCA. Further analyses and post-estimations including all biomarkers were consequently not conducted.

We assessed the predictive capability of the principal components identified by fitting a logistic regression model to estimate the likelihood of CMD defined as a binary classifier, with CFVR <2.0 designating CMD. A basic model including age and all baseline variables significantly related to CFVR was compared to a model adding any significant principle components, and the difference in diagnostic ability was evaluated using comparative receiver operating characteristics. Statin use was additionally included in the logistic regression model to assess potential anti-inflammatory effects related to statins.

Further, for the principal components which were significantly related to CFVR after multivariate adjustment, each individual biomarker was also separately compared to the basic model, along with all biomarkers which were related to CFVR in linear regression with $p < 0.01$.

All analyses were performed using STATA/IC 13.1 (StataCorp LP, College Station, TX, USA).

2.5. Ethics

The study was performed in accordance with the Helsinki Declaration and was approved by the Danish Regional Committee on Biomedical Research Ethics (H-3-2012-005). All participants gave written informed consent, after receiving oral and written information about the study.

3. Results

3.1. Study population

The study population consisted of 174 women with angina, median age was 64 years (SD 9.8), median CFVR was 2.3 (IQR 1.9–2.7), and 28% of patients had CFVR < 2.0. Patients with low CFVR more often had diffuse atherosclerosis in the baseline coronary angiogram ($p < 0.001$) and had more hypertension ($p = 0.01$) than patients with higher CFVR, while no other baseline characteristics were associated with CFVR ($p > 0.05$) (Online Fig. 2).

3.2. Biomarkers

95% of the measured proteins were detected in all 174 samples (See Supplemental Materials). In univariate regression analysis, 18 of the 92 protein biomarkers were significantly related to CFVR (Table 1).

3.3. PCA

Evaluation of the data structure generally supported the use of PCA: The Pearson correlation matrix showed a large proportion of significant correlations between the selected 18 biomarkers, and roughly half of the correlation coefficients were >0.5 (Online Fig. 3). The Kaiser-Meyer-Olkin measures of sampling adequacy were high (Online Fig. 4), and Bartlett's test for sphericity was highly significant ($\chi^2 < 0.001$). In the initial PCA output, 3 components had eigenvalues >1.2, supporting selection of 3 components for further loading structure interpretation (Table 2).

The component loading structure was examined in a loading matrix to assess biomarker loading patterns on the 3 selected components. Differential loading (i.e. one variable loading significantly to more than one component) was minimal (Table 2). The first component (PC1) had loadings >0.25 for 8 biomarkers: C-C motif ligand 16 (CCL16), C-X-C motif ligand 16 (CXCL16), growth differentiation factor 15 (GDF15), peptidoglycan recognition protein 1 (PGLYRP1), ST2, tumor necrosis factor receptor-1 (TNFR1), soluble urokinase-type plasminogen activator receptor (suPAR) and tumor necrosis factor receptor superfamily member 10C (TNFRSF10C); the second component (PC2) for 8 biomarkers:

Table 1
Protein biomarkers significantly associated with CFVR.

Biomarker	R	p Value	Biomarker	R	p Value
CCL15	-0.16	0.034	NTproBNP	-0.16	0.036
CCL16	-0.15	0.046	PGLYRP1	-0.20	0.009
CHI3L1	-0.19	0.011	PON3	0.16	0.037
CHIT1	-0.18	0.020	SCGB3A	-0.16	0.034
CXCL16	-0.17	0.029	ST2	-0.16	0.038
GDF15	-0.25	0.001	suPAR	-0.23	0.003
Gal4	-0.17	0.026	TNFR1	-0.15	0.047
MMP3	-0.15	0.045	TNFRSF10C	-0.19	0.012
MMP9	-0.24	0.002	tPA	-0.16	0.036

Regression analysis results for the relation between significant biomarkers CFVR value. R = Pearson correlation coefficient.

Biomarker abbreviations alphabetically: CCL15 = C-C motif ligand 15, CCL16 = C-C motif ligand 16, CHI3L1 = Chitinase-3-like protein 1, CHIT1 = chitotriosidase, CXCL16 = C-X-C motif ligand 16, GDF15 = growth differentiation factor 15, Gal4 = Galectin-4, MMP3 = matrix metalloproteinase 3, MMP9 = matrix metalloproteinase 9, NTproBNP = N-terminal pro-hormone of brain natriuretic peptide, PGLYRP1 = peptidoglycan recognition protein 1, PON3 = paraoxonase 3, SCGB3A = secretoglobulin family 3a member 2, TNFR1 = tumor necrosis factor receptor 1.

Table 2
Principal component analysis loading matrix.

Variable	PC1	PC2	PC3
CCL15	0.244	-0.258	-0.016
CCL16	0.258	-0.227	0.054
CHI3L1	0.218	-0.088	-0.275
CHIT1	0.142	-0.043	0.100
CXCL16	0.291	-0.001	0.193
GDF15	0.277	-0.172	-0.135
Gal4	0.228	-0.257	-0.050
MMP3	0.247	0.017	0.280
MMP9	0.224	0.433	-0.290
NTproBNP	0.151	-0.285	0.120
PGLYRP1	0.253	0.314	-0.288
PON3	0.133	0.224	0.686
SCGB3A	0.150	0.347	0.188
ST2	0.252	-0.115	0.194
TNFR1	0.305	-0.038	-0.023
TNFRSF10C	0.254	0.258	-0.020
suPAR	0.294	0.249	-0.156
tPA	0.211	-0.301	-0.148
Eigenvalue	9.18	1.47	1.21
Total variance, %	51.04	8.18	6.73
Accumulative variance, %	51.04	59.22	65.95

Upper part: Loading scores for the 3 retained principal components. Lower part: Eigenvalues and corresponding variance for the 3 components.

PC = principal component. Biomarker abbreviations as for Table 1. Bold highlights loadings >0.25.

C-C motif ligand 15 (CCL15), galectin-4 (Gal4), MMP-9, N-terminal pro-hormone of brain natriuretic peptide (NTproBNP), PGLYRP1, secretoglobulin family 3a member 2 (SCGB3A2), TNFRSF10C and tissue plasminogen activator (tPA); and the third component (PC3) for 5 biomarkers: Chitinase-3-like protein 1 (CHI3L1), matrix metalloproteinase 3 (MMP-3), MMP-9, PGLYRP1 and paraoxonase 3 (PON3). Total variance explained by the 3 components was 66%.

The aggregate component scores calculated from the component loading coefficients for each patient were significantly associated with CFVR for PC1 ($p = 0.002$) and PC3 ($p = 0.009$) in univariate regression analysis, while PC2 showed no association with CFVR ($p = 0.91$). After multivariate adjustment for CMD risk factors (Online Fig. 5) component 1 was still significantly related to CFVR ($p = 0.04$), while component 3 was not ($p = 0.14$).

Addition of PC1 scores to a multiple logistic regression model for predicting CFVR < 2.0 did not significantly improve area under the curve (0.67 vs 0.64, $p = 0.22$) (Fig. 1). Further addition of statin use to

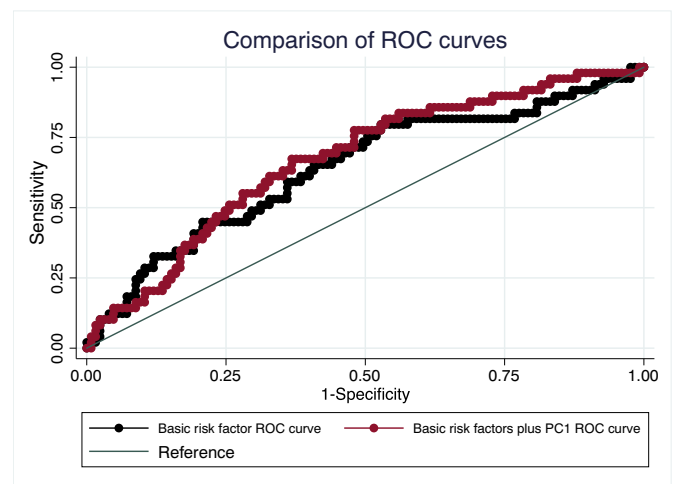


Fig. 1. Comparison of ROC curves Addition of PC1 to the ROC plot increased predictive power (red curve), but this effect was not significant ($p = 0.22$). PC1 = principal component 1.

the logistic regression model neither increased nor decreased the area under the curve significantly (0.64 vs. 0.67, $p = 0.24$).

Individual addition of the PC1 related biomarkers and MMP-9 to the basic model did not significantly improve the area under the curve (Online Fig. 6).

4. Discussion

In this study investigating the relationship between a large number of CVD protein biomarkers and CMD, 18 biomarkers were significantly related to CFVR. We identified two principal components significantly associated with CFVR. PC1 accounted for 51% of biomarker variance and was significantly related to CFVR after multiple adjustment.

4.1. Principal component 1 protein biomarkers and inflammation

The 6 PC1 biomarkers CCL16, CXCL16, PGLYRP1, TNFR1, GDF15 and TNFRSF10C all have direct or indirect associations with the interleukin IL-1 β – TNF- α – IL-6 – CRP inflammatory pathway and the pro-inflammatory hypothesis of atherosclerotic development. This is noteworthy in the light of past findings in CMD research which also highlight this specific pathway [13,14]. In Fig. 2, an overview of functional relationships between PC1 biomarkers and related proteins are outlined graphically.

The chemokine C-C motif ligand 16 (CCL16) is upregulated in stress conditions, and elicits its effects both as a direct chemoattractant for

monocytes and lymphocytes, and by activating the more well described chemokine C-C motif ligand 2 (CCL2) which has been implicated in development of cardiac fibrosis, inflammation and hypertension in animal models [15]. The related chemokine CXCL16 (CXCL16) also induces CCL2 release, but more importantly CXCL16 is an important inflammatory mediator in the IL-1 β /TNF- α inflammatory pathway in patients with atherosclerotic heart disease [16]. In CAD patients blockage of IL-1 β signaling directly inhibited CXCL16 release from peripheral blood monocytes [17]. CXCL16 is expressed in atherosclerotic lesions in both humans and animal models and has also been proposed as a mediator between inflammation and dyslipidemia in the atherosclerotic process due to increased foam cell formation resulting from CXCL16 stimulation [18].

Peptidoglycan is a major constituent of most bacteria's cell wall, and peptidoglycan recognition proteins (PGLYRPs) play an important role both as direct bactericides and as inflammatory mediators. Presence of PGLYRP1 has been demonstrated in human coronary atheroma, stimulating production of the pro-inflammatory cytokines interleukin-1/-6, TNF- α , CCL2 and metalloproteinases such as MMP-9 [19]. Increased levels of PGLYRP1 were associated with both increased coronary artery calcium and plaque burden in the Dallas Heart Study of >3000 community dwelling individuals [20]. In a subsequent follow-up study PGLYRP1 levels robustly predicted a 3-fold increase in atherosclerotic CVD events, and this association was strongest in patients with elevated hs-CRP levels. Accordingly, PGLYRP1 has been proposed as a possible link between

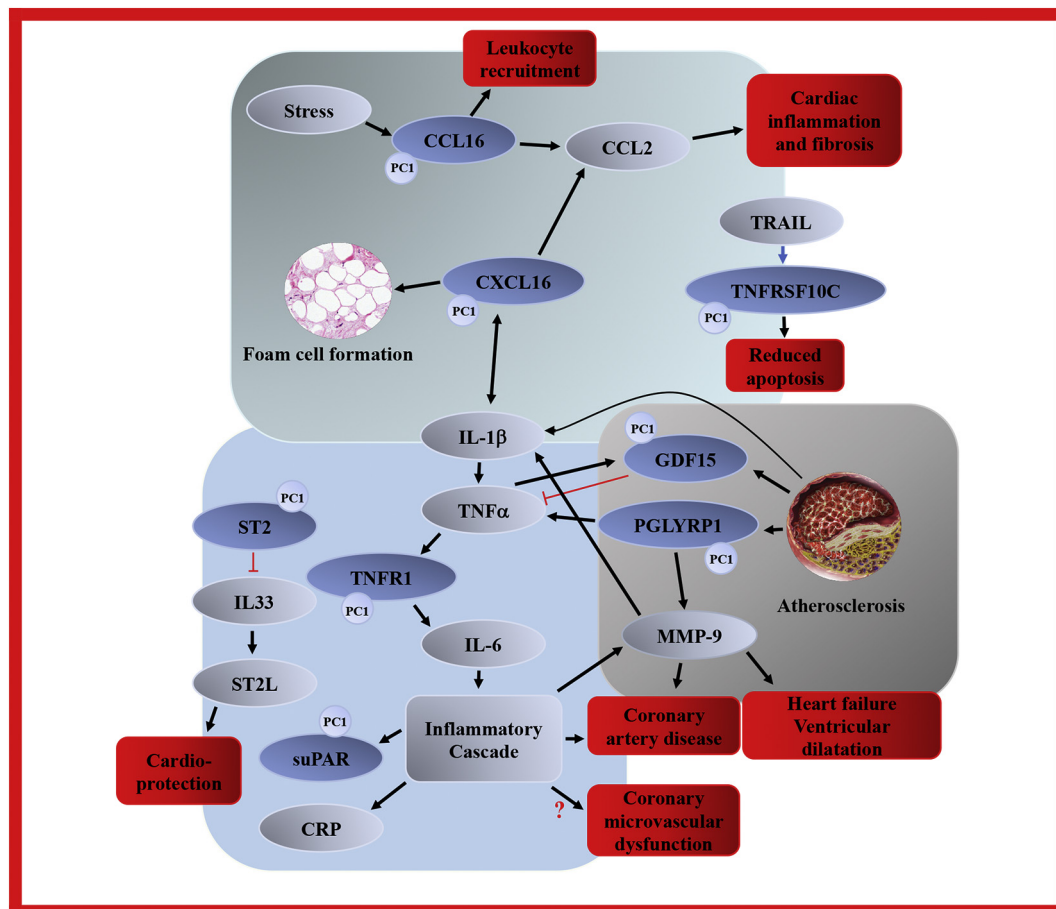


Fig. 2. Principal component 1 biomarker overview Protein biomarker interactions and downstream pathological effects. See “Discussion” for details and references. Principal component 1 biomarkers in grey, highlighted with “PC1” marker. Other proteins and effectors in dark blue. Downstream effects in red. MMP-9 was not part of PC1, but was independently related to CFVR after multivariate adjustment. Black arrows indicate a stimulation or upregulation, red truncated connectors indicate inhibition. Red question mark indicates a possible connection. CCL2 = C-C motif ligand 2, CRP = c-reactive protein, IL-1 β = interleukin 1 β , IL-6 = interleukin 6, IL-33 = interleukin 33, ST2L = transmembrane ST2, TNF α = tumor necrosis factor α , TRAIL = TNF-related apoptosis-inducing ligand. All other abbreviations as in Table 1.

subclinical inflammation, immuno-modulation and early development of atherosclerosis.

While TNF- α itself was not featured in the present biomarker panel due to technical restrictions in the applied assay, its main effector receptor TNF receptor 1 (TNFR1) was significantly associated with CFVR and showed high loading on PC1. Activation of TNFR1 produces extensive downstream pro-inflammatory effects e.g. activation of IL-6 cascade and nuclear factor kappa B [21]. Downstream harmful inflammatory effects of TNFR1 stimulation have long been implicated in CAD development through alteration of vascular cell function [22]. TNFR1 activation is also suggested to play a more short-acting role in the event of acute coronary syndrome plaque instability, and elevated levels of TNF- α and TNFR1 have been shown to predict coronary events in 1464 patients with chronic systolic heart failure [23]. A range of pathways associated with TNF- α , TNFR1, IL-6 and IL-1 β are being investigated for possible therapeutic inhibition which may improve outcomes in different groups of CVD patients [22,24], e.g. the TNF- α inhibitors adalimumab and infliximab [25]. It has recently been demonstrated in the CANTOS trial that monoclonal antibodies targeting interleukin-1 β , an upstream regulator of TNF- α , reduced first occurrence of major cardiovascular events (myocardial infarction (MI), stroke and CV death) compared with placebo in patients with previous MI [26].

TNF receptor superfamily 10C (TNFRSF10C) is also known as decoy receptor 1, and TNF-related apoptosis-inducing ligand (TRAIL) is the primary ligand for TNFRSF10C. The main known effect of TRAIL is to induce apoptosis via binding to death receptors, but binding of TRAIL to TNFRSF10C conversely attenuates the apoptotic actions of TRAIL via sequestering [27]. The exact role of TNFRSF10C is largely unknown, but it may be speculated that a general inflammatory activation in CMD patients also upregulates balancing proteins such as TNFRSF10C, which in turn may moderate the cascade responses of the pro-inflammatory pathways.

Growth differentiation factor 15 (GDF15) is a further inflammatory biomarker which showed loading on PC1. It has been intensively studied in recent years as a strong and robust predictor of all-cause mortality, and adverse cancer and CVD outcomes [28]. GDF15 synthesis is stimulated by TNF- α , but at the same time it may also serve as a paracrine negative feedback inhibitor of TNF- α release which moderates the TNF- α cascade. GDF15 is highly expressed by macrophages in atherosclerotic lesions, and GDF15 has shown promise as a new, robust predictor for adverse CVD outcomes in large observational cohorts and clinical trials such as STABILITY and PLATO [29,30].

Past biomarker studies in patients with CMD have also implicated TNF- α , IL-6 and hs-CRP as possible pathogenetic determinants: A study of 86 obese patients found IL-6 and TNF- α to be significantly associated with CMD ($p < 0.03$ for both) [14], and in a small population of cardiac syndrome X patients hs-CRP was significantly associated with CMD ($p = 0.001$) [13].

4.1.1. ST2

ST2 is also known as interleukin-1 receptor-like 1 and exists in a membrane bound receptor form (ST2L) and a soluble form (sST2); the latter was measured in the present study and found to load on PC1. The ligand for both forms is interleukin-33 (IL-33), and in a number of experimental studies myocardial mechanical stress and hypoxia have been shown to upregulate the expression of both IL-33 and ST2L, resulting in cardioprotective effects such as reduction of cardiomyocyte hypertrophy, prevention of hypoxia-related apoptosis and reduced mortality; the soluble form sST2, on the other hand, sequesters IL-33 thereby decreasing activation of membrane bound ST2L and consequently reducing the given cardioprotective effects (Fig. 2) [31]. While IL-33 significantly reduced atherosclerotic plaque size in experimental models of atherosclerosis, addition of sST2 directly counteracted this effect and resulted in increased plaque size [32]. These findings have motivated numerous studies examining the potential of sST2 as a risk marker in patients with heart failure, acute coronary syndrome

(ACS) and stable CAD [33,34], persistently demonstrating sST2 as a robust prognostic biomarker of CVD outcomes. Therapeutic trials with sST2-inhibiting antibodies have been shown to reduce graft versus host disease severity and mortality, while sST2-inhibition remains to be investigated in CVD.

4.1.2. Soluble urokinase-type plasminogen activator receptor (suPAR)

SuPAR loaded on PC1, and was also highly significantly related to CFVR as a separate biomarker ($p = 0.003$). Increased suPAR levels reflect low grade inflammation and have been linked to the early stages of atherosclerotic development and subclinical organ damage [35]. SuPAR has previously been linked to CMD assessed by intracoronary Doppler flow measurement of CMD in 66 patients with non-obstructive coronary atherosclerosis, where a higher level of suPAR remained significantly associated with a lower CFVR after multivariate adjustment ($p = 0.04$) [36]. SuPAR level was further related to the presence of CAD and CVD outcomes in >3000 patients with suspected CAD, and addition of suPAR improved the C-statistic significantly compared to a model based on traditional risk factors [37].

4.1.3. Common features of PC1 related biomarkers

Most of the 8 protein biomarkers with high loadings on PC1 are related to inflammatory pathways, either as upstream regulators, downstream protein products expressing a pro-inflammatory state, or parallel and moderating messenger pathways (Fig. 2). A majority of the biomarkers have connection with the important IL-1 β - TNF- α - IL-6 - CRP inflammatory activation pathway, and inflammatory biomarkers such as GDF15, ST2 and suPAR are increasingly discussed as promising future risk stratification biomarkers in both stable CAD, ACS and heart failure. The concept of prolonged low-grade subclinical inflammation as a pivotal component in the development of CVD is not new [38], but in recent years evidence supporting the inflammatory hypothesis has been gathering [39]. This is further supported by biomarker studies which have helped introduce potential upstream proteins available for targeted treatment interventions. Several studies are evaluating different immunomodulation strategies in CVD [25,26], yet no such interventional trials have been undertaken in the field of CMD. The current study suggests that the well-known association between inflammation and macrovascular CVD may also pertain to presence of CMD, i.e. inflammation may be a common pathogenetic factor in both macro- and microvascular disease.

Addition of PC1 to a standard risk factor based prediction model did not significantly improve the area under the curve. Consequently, the PCA results are not readily translatable to diagnostic implementation, and should be interpreted as hypothesis generating. Lack of enhancement of predictive power after adding a biomarker to standard risk factors has also been described for a majority of CVD biomarkers in past, larger studies. This finding has been attributed to a possible role of biomarkers as mediating or co-incident factors, rather than entities representing independent increases in patient risk [40,41].

4.2. Other protein biomarkers

MMP-9 had a strong association with CFVR ($p = 0.002$) and is also activated by PGLYRP1 which was part of PC1. Matrix metalloproteinases (MMPs) are important in normal degradation of collagen in the extracellular matrix and migration of neutrophil granulocytes across the basement membrane. Following stress such as MI, MMPs are activated by pro-inflammatory cytokines resulting in increased breakdown of the cardiomyocyte collagen skeleton which otherwise ensures normal structure, thereby contributing to post-ischemic ventricular dilatation [42]. MMP-9 has been studied in relation to development of heart failure, where MMP-9 levels were associated with increased left ventricular diastolic dimensions and wall thickness in 699 patients with previous MI [43]. MMP-9 has also shown potential as a general CAD risk marker,

e.g. predicting CV events in >1000 patients with baseline CAD [44]. MMP-9's direct involvement in heart failure remodeling and cytokine activation has given rise to interventional trials with the nonspecific MMP inhibitor doxycycline, effectively blocking MMP-9 activities and attenuating myocardial fibrosis and adverse LV remodeling [45].

In a previous, smaller study by our group investigating possible associations between the CVD III biomarker panel and CMD evaluated with PET, 4 biomarkers were significantly associated with CMD, 3 of which (GDF15, tPA, Gal4) were also significant in the present study [3]. Inter-study differences may conceivably be ascribed to smaller population size, different examination modalities and random variation. Few other studies have investigated biomarkers in small CMD populations: One study found increased activation of circulating progenitor cells related to CMD in 123 women [46], while a study by Bozcali et al. in 115 cardiac syndrome X patients found higher levels of the inflammation and fibrosis biomarker galectin-3 (Gal3) compared with healthy controls [47]. In the present study, there was no significant relation between Gal3 and CFVR.

4.3. Study strengths and limitations

To date only few studies have examined protein biomarkers in CMD, and they have mainly evaluated individual biomarkers in small patient populations. The present study is the first to evaluate a large-scale biomarker panel in relation to CMD. Further, we attempt to uncover new internal patterns of CMD-related biomarkers through the utilization of PCA statistics, which is an apt approach for identification of underlying patterns in large data sets.

Although the present study is fairly large in the context of CMD research, the main limitation is nonetheless the relatively small population sample size in relation to the wide range of biomarkers represented in the used assay. As noted, strict adjustment of *p*-values for multiple comparisons was not possible due to population size (risk of type II error), and this in turn naturally introduces a risk of type I error in the initial separate regression analyses. Accordingly, our findings should be interpreted as hypothesis generating; however, the use of PCA and subsequent review of available evidence support the notion that a considerable proportion of the identified correlations are not mere chance findings, since 18 out of 92 biomarkers were related to CFVR, and the identified PC1 biomarkers share roles in common inflammatory pathways.

The method used for inducing maximum hyperemia by dipyridamole infusion mainly evaluates non-endothelium dependent vasodilatation. Results are not comparable to coronary vascular function assessed by e.g. acetylcholine provocation.

All studied patients were female, and it is therefore unknown whether the results would be different in a cohort including male angina patients.

4.4. Conclusion

We identified 18 protein biomarkers significantly associated with impaired microvascular dilatation in women with angina but no significant CAD. We further identified 8 biomarkers with common loading on a principal component significantly associated with CMD after multiple adjustment, most of which were related to the TNF- α – IL-6 pro-inflammatory pathway.

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Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jjcha.2019.100370>.

References

- [1] N.D. Mygind, M.M. Michelsen, A. Pena, D. Frestad, N. Dose, A. Aziz, et al., Coronary microvascular function and cardiovascular risk factors in women with angina pectoris and no obstructive coronary artery disease: the iPOWER study, *J. Am. Heart Assoc.* 5 (3) (2015), e003064.
- [2] C. Noel Bairey Merz, C.J. Pepine, M.N. Walsh, J.L. Fleg, Ischemia and no obstructive coronary artery disease (INOCA): developing evidence-based therapies and research agenda for the next decade, *Circulation*. 135 (11) (2017) 1075–1092.
- [3] J. Schroder, R. Zethner-Moller, K.B. Bové, N.D. Mygind, P. Hasbak, M.M. Michelsen, et al., Protein biomarkers and coronary microvascular dilatation assessed by rubidium-82 PET in women with angina pectoris and no obstructive coronary artery disease, *Atherosclerosis*. 275 (2018 Jun 19) 319–327.
- [4] E. Prescott, S.Z. Abildstrøm, A. Aziz, N.B. Merz, I. Gustafsson, J. Halcox, et al., Improving diagnosis and treatment of women with angina pectoris and microvascular disease: the iPOWER study design and rationale, *Am. Heart J.* 167 (4) (2014) 452–458.
- [5] R. Sicari, P. Nihoyannopoulos, A. Evangelista, J. Kasprzak, P. Lancellotti, D. Poldermans, et al., Stress echocardiography expert consensus statement: European Association of Echocardiography (EAE) (a registered branch of the ESC), *Eur. J. Echocardiogr.* 9 (4) (2008 Feb 19) 415–437.
- [6] E. Assarsson, M. Lundberg, G. Holmquist, J. Björkstén, S.B. Thorsen, D. Ekman, et al., Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability, *PLoS One* 9 (4) (2014).
- [7] G. Norman, D. Streiner, *Biostatistics: The Bare Essentials*, 3rd ed. Jama. pmph, 2009.
- [8] J.N. Luke, A.D. Brown, L. Brazionis, K. O'Dea, J.D. Best, R.A. McDermott, et al., Exploring clinical predictors of cardiovascular disease in a central Australian aboriginal cohort, *Eur. J. Prev. Cardiol.* 20 (2) (2013) 246–253.
- [9] E. Goodman, L.M. Dolan, J.A. Morrison, S.R. Daniels, Factor analysis of clustered cardiovascular risks in adolescence: obesity is the predominant correlate of risk among youth, *Circulation*. 111 (15) (2005) 1970–1977.
- [10] A. Giuliani, The application of principal component analysis to drug discovery and biomedical data, *Drug Discov. Today* 22 (7) (2017) 1069–1076.
- [11] J. Nunnally, I. Bernstein, *Psychometric Theory*. Vol. 3, McGraw-Hill, New York, 1994 701.
- [12] Stevens JP. *Applied multivariate statistics for the social sciences*. Routledge. 2009.663 p.
- [13] A. Recio-Mayoral, O.E. Rimoldi, P.G. Camici, J.C. Kaski, Inflammation and microvascular dysfunction in cardiac syndrome X patients without conventional risk factors for coronary artery disease, *JACC Cardiovasc. Imaging* 6 (6) (2013 Jun) 660–667.
- [14] F. Tona, R. Serra, L. Di Ascenzo, E. Osto, A. Scarda, R. Fabris, et al., Systemic inflammation is related to coronary microvascular dysfunction in obese patients without obstructive coronary disease, *Nutr. Metab. Cardiovasc. Dis.* 24 (4) (2014 Apr) 447–453.
- [15] J.Z. Shen, J. Morgan, G.H. Tesch, P.J. Fuller, M.J. Young, CCL2-dependent macrophage recruitment is critical for mineralocorticoid receptor-mediated cardiac fibrosis, inflammation, and blood pressure responses in male mice, *Endocrinology*. 155 (3) (2014) 1057–1066.
- [16] J. Xing, Y. Liu, T. Chen, Correlations of chemokine CXCL16 and TNF- α with coronary atherosclerotic heart disease, *Exp. Ther. Med.* 15 (1) (2018) 773–776.
- [17] C. Smith, B. Halvorsen, K. Otterdal, T. Wæhre, A. Yndestad, B. Fevang, et al., High levels and inflammatory effects of soluble CXCL16 in coronary artery disease: Down-regulatory effects of statins, *Cardiovasc. Res.* 79 (1) (2008) 195–203.
- [18] D.M. Wuttge, X. Zhou, Y. Sheikine, D. Wågsäter, V. Stemme, U. Hedin, et al., CXCL16/SR-PSOX is an interferon- γ -regulated chemokine and scavenger receptor expressed in atherosclerotic lesions, *Arterioscler. Thromb. Vasc. Biol.* 24 (4) (2004) 750–755.
- [19] J.E. Wang, P.F. Jørgensen, M. Almlöf, C. Thiemermann, S.J. Foster, A.O. Aasen, et al., Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model, *Infect. Immun.* 68 (7) (2000) 3965–3970.
- [20] A. Rohatgi, C.R. Ayers, A. Khera, D.K. McGuire, S.R. Das, S. Matulevicius, et al., The association between peptidoglycan recognition protein-1 and coronary and peripheral atherosclerosis: observations from the Dallas Heart Study, *Atherosclerosis*. 203 (2) (2009) 569–575.
- [21] L. Cabal-Hierro, P.S. Lazo, Signal transduction by tumor necrosis factor receptors, *Cell. Signal.* 24 (6) (2012) 1297–1305.
- [22] P. Kleinbongard, G. Heusch, R. Schulz, TNF α in atherosclerosis, myocardial ischemia/reperfusion and heart failure, *Pharmacol. Ther.* 127 (3) (2010) 295–314.
- [23] S.H. Nymo, J. Hulthe, T. Ueland, J. McMurray, J. Wikstrand, E.T. Askevold, et al., Inflammatory cytokines in chronic heart failure: Interleukin-8 is associated with adverse outcome. Results from CORONA, *Eur. J. Heart Fail.* 16 (1) (2014) 68–75.
- [24] P.M. Ridker, From C-reactive protein to Interleukin-6 to Interleukin-1: moving upstream to identify novel targets for atheroprotection, *Circ. Res.* 118 (1) (2016) 145–156.
- [25] P.M. Ridker, T.F. Lüscher, Anti-inflammatory therapies for cardiovascular disease, *Eur. Heart J.* 35 (27) (2014) 1782–1791.

- [26] Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med*. 2017; *NEJMoa1707914*.
- [27] A. Bisgin, E. Terzioğlu, C. Aydin, B. Yoldas, V. Yazisiz, N. Balci, et al., TRAIL death receptor-4, decoy receptor-1 and decoy receptor-2 expression on CD8⁺ T cells correlate with the disease severity in patients with rheumatoid arthritis, *BMC Musculoskelet. Disord.* 11 (2010).
- [28] K.C. Wollert, T. Kempf, L. Wallentin, Growth differentiation factor 15 as a biomarker in cardiovascular disease, *Clin. Chem.* 63 (1) (2017) 140–151.
- [29] E. Hagström, C. Held, R.A.H. Stewart, P.E. Aylward, A. Budaj, C.P. Cannon, et al., Growth differentiation factor 15 predicts all-cause morbidity and mortality in stable coronary heart disease, *Clin. Chem.* 63 (1) (2017) 325–333.
- [30] E. Hagström, S.K. James, M. Bertilsson, R.C. Becker, A. Himmelmann, S. Husted, et al., Growth differentiation factor-15 level predicts major bleeding and cardiovascular events in patients with acute coronary syndromes: results from the PLATO study, *Eur. Heart J.* 37 (16) (2016) 1325–1333.
- [31] E.O. Weinberg, M. Shimpf, G.W. De Keulenaer, C. MacGillivray, Tominaga S. ichi, S.D. Solomon, et al., Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction, *Circulation.* 106 (23) (2002) 2961–2966.
- [32] Seki K, Sanada S, Kudinova AY, Steinhilber ML, Handa V, Gannon J, et al. Interleukin-33 prevents apoptosis and improves survival after experimental myocardial infarction through ST2 signaling. *Circ Hear Fail.* 2009; nov (6): 684–91.
- [33] B. Dieplinger, M. Egger, M. Haltmayer, M.E. Kleber, H. Scharnagl, G. Silbernagel, et al., Increased soluble ST2 predicts long-term mortality in patients with stable coronary artery disease: results from the Ludwigshafen risk and cardiovascular health study, *Clin. Chem.* 60 (3) (2014) 530–540.
- [34] A.M. Richards, S. Di Somma, T. Mueller, ST2 in stable and unstable ischemic heart diseases, *Am. J. Cardiol.* 115 (7) (2015) 48B–58B.
- [35] S. Lyngbæk, T. Sehested, J.L. Marott, T.W. Hansen, M.H. Olsen, O. Andersen, et al., CRP and suPAR are differently related to anthropometry and subclinical organ damage, *Int. J. Cardiol.* 167 (3) (2013) 781–785.
- [36] G. Mekonnen, M.T. Corban, O.Y. Hung, P. Eshtehardi, D.J. Eapen, H. Al-Kassem, et al., Plasma soluble urokinase-type plasminogen activator receptor level is independently associated with coronary microvascular function in patients with non-obstructive coronary artery disease, *Atherosclerosis.* 239 (1) (2015 Mar) 55–60.
- [37] D.J. Eapen, P. Manocha, N. Ghasemzadeh, R.S. Patel, Kassem H. Al, M. Hammadah, et al., Soluble urokinase plasminogen activator receptor level is an independent predictor of the presence and severity of coronary artery disease and of future adverse events, *J. Am. Heart Assoc.* 3 (5) (2014).
- [38] P. Libby, P.M. Ridker, A. Maseri, Inflammation and atherosclerosis, *Circulation.* 105 (2002) 1135–1143.
- [39] G.R. Geovanini, P. Libby, Atherosclerosis and inflammation: overview and updates, *Clin. Sci.* 132 (12) (2018) 1243–1252.
- [40] J. Yeboah, R.L. McClelland, T.S. Polonsky, G.L. Burke, C.T. Sibley, D.O. Leary, et al., Comparison of novel risk markers for improvement in cardiovascular risk assessment in intermediate risk individuals. The multi-ethnic study of atherosclerosis, *JAMA* August. 229624 (3088) (2012) 788–795.
- [41] J. Macnamara, D.J. Eapen, A. Quyyumi, L. Sperling, Novel biomarkers for cardiovascular risk assessment: current status and future directions, *Futur. Cardiol.* 11 (5) (2015) 597–613.
- [42] E. Braunwal, Biomarkers in heart failure management, *Curr. Opin. Cardiol.* 23 (2) (2008) 127–133.
- [43] J. Sundström, J.C. Evans, E.J. Benjamin, D. Levy, M.G. Larson, D.B. Sawyer, et al., Relations of plasma matrix metalloproteinase-9 to clinical cardiovascular risk factors and echocardiographic left ventricular measures: the Framingham heart study, *Circulation.* 109 (23) (2004) 2850–2856.
- [44] S. Blankenberg, H.J. Rupprecht, O. Poirier, C. Bickel, M. Smieja, G. Hafner, et al., Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease, *Circulation.* 107 (12) (2003) 1579–1585.
- [45] G. Cerisano, P. Buonamici, R. Valenti, R. Sciagrà, S. Raspanti, A. Santini, et al., Early short-term doxycycline therapy in patients with acute myocardial infarction and left ventricular dysfunction to prevent the ominous progression to adverse remodeling: the TIPTOP trial, *Eur. Heart J.* 35 (3) (2014) 184–191.
- [46] G. Mekonnen, S.S. Hayek, P.K. Mehta, Q. Li, E. Mahar, L. Mou, et al., Circulating progenitor cells and coronary microvascular dysfunction: results from the NHLBI-sponsored Women's ischemia syndrome evaluation-coronary vascular dysfunction study (WISE-CVD), *Atherosclerosis.* 253 (2016 Oct) 111–117.
- [47] E. Bozcali, V. Polat, G. Aciksari, S. Opan, I.H. Bayrak, N. Paker, et al., Serum concentrations of galectin-3 in patients with cardiac syndrome X, *Atherosclerosis.* 237 (1) (2014 Nov) 259–263.