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Original Article

Plasma proteomic analysis of association between atrial fibrillation, coronary microvascular disease and heart failure

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Abstract: The clinical association between atrial fibrillation (AF), coronary microvascular disease (CMD) and heart failure with preserved ejection fraction (HFpEF) is highly prevalent, however the mechanism behind this association is not known. We hypothesized that plasma proteomic analysis can identify novel biomarkers and the mechanistic pathways in concomitant AF, CMD and HFpEF. To discover circulating biomarkers for the association between AF, CMD and HFpEF, an unbiased label-free quantitative proteomics approach was used in plasma derived from patients who underwent coronary physiology studies (n=18). Circulating proteins were analyzed by liquid chromatography-mass spectrometry and screened to determine candidate biomarkers of the concomitant AF, CMD and HFpEF. We identified 130 dysregulated proteins across the groups with the independent patient replicates. Among those, 35 proteins were candidate biomarkers of the association between AF, CMD and HFpEF. We found significantly elevated SAA1, LRG1 and APOC3 proteins in the coexistence of AF, CMD and HFpEF, whereas LCP1, PON1 and C1S were markedly downregulated in their associations. AF was associated with reduced LCP1, KLKB1 and C4A in these patients. Combined downregulation of PON1 and C1S was a marker of concurrent HFpEF and CMD. PON1 was associated with HFpEF while C1S was a marker of CMD. These proteins are related to inflammation, extra cellular remodeling, oxidative stress, and coagulation. In conclusion, plasma proteomic profile provides biomarkers and mechanistic insight into the association of AF, CMD and HFpEF. SAA1, LRG1, APOC3, LCP1, PON1 and C1S are candidate markers for the risk stratification of their associations and potential underlying mechanistic pathways.

Keywords: Atrial fibrillation, coronary microvascular dysfunction, heart failure, biomarkers, proteomics

Introduction

Atrial fibrillation (AF) commonly occurs in patients with heart failure with preserved ejection fraction (HFpEF) [1, 2]. The coexistence of AF and HFpEF is associated with a higher risk of stroke, hospitalization, dementia, kidney failure, myocardial infarction and death compared to those with HFpEF or AF alone [3-5]. Coronary microvascular dysfunction (CMD) is a new frontier in cardiovascular disease with a known association with HFpEF [6-9]. Studies demonstrated a high incidence of CMD in patients with HFpEF and a likely association with AF [8-11]. Recently, we found that CMD is highly prevalent in patients with AF with or without HFpEF. It is also associated with poor clinical outcome [12]. CMD with AF or HFpEF is associated with higher risk of mortality and HF hospitalization [12]. This has a major clinical implication. Risk stratification is therefore important to develop treatment and/or preventive strategies for the patients with CMD, AF and HFpEF.

Circulating biomarkers are essential for risk stratification, understanding of the mechanisms of the disease progression, and ultimately the identification of novel therapeutic targets [13, 14]. The mass spectrometry (MS)-based proteomics approach is a rapidly evolving technique for biomarker discovery in clinical samples. It can determine the role of distinct biological pathways in a disease process. Thus far, no biological markers for AF, CMD, and HFpEF have been adopted in clinical practice, though a few studies have identified altered proteins [13-

16]. Also, the mechanisms of their association have not been characterized. Our recent studies in both mouse and human atria demonstrated progressive atrial remodeling in the coexistence of AF and HF [17-19]. This was associated with biatrial enlargement, a reduced cardiomyocyte cell population, patchy fibrosis, inflammation, oxidative stress and metabolic dysregulation. CMD was reported to contribute to HFpEF pathophysiology through cardiomyocyte stiffening, diffuse interstitial fibrosis and myocardial ischemia [6, 11]. Proteomic profiles in the relationship between these three entities can reveal pathological processes in association with disease pathogenesis. Thus, circulating proteins can identify markers of the pathways mechanistically related to the disease association and clinically useful predictors for risk stratification.

We studied the plasma samples from the patients with AF, CMD and/or HFpEF to identify dysregulated protein as potential biomarkers of their association. Liquid chromatography-mass spectrometry (LC-MS) based untargeted and label-free quantification (LFQ) proteomic analysis was performed. Circulating plasma proteins from patients with CMD, HFpEF and AF were screened and the candidate biomarkers of their association were determined. Also, dysregulated proteins were evaluated to characterize the mutual mechanistic pathways in these disease processes and their coexistence.

Methods

Study samples

Plasma samples were obtained from the patients who underwent invasive coronary physiology evaluation at the University of Chicago Medical Center (UCMC) from January 2018 to April 2019. In order to eliminate selection biases, all consecutive patients with angina or dyspnea who were referred to the cardiac catheterization laboratory for invasive angiography and the coronary physiology studies were included in this study. A written informed consent was obtained from each patient. The study was approved by the UCMC Institutional Review Board (#IRB14-0927). Following coronary angiography, if there was no obstructive coronary artery disease defined as a stenosis >50% in the left main coronary artery, >70% in a non-left main coronary artery, or any stenosis with a fractional flow reserve of ≤0.80, an invasive coronary physiology study was performed [12]. Coronary angiography, and coronary physiology studies were performed by using a standardized protocol as described previously [9, 12]. CMD was defined as abnormal coronary flow reserve (CFR <2.0) in the absence of obstructive coronary artery disease [6, 9]. Clinical data were abstracted from a centralized electronic medical record containing complete records of patients treated and followed at the UCMC. Cardiac function and structure including left ventricular ejection fraction (LVEF) was evaluated by echocardiography within 90 days of the procedure. HFpEF was diagnosed if the patient had a LVEF ≥50%, met Framingham criteria for HF and had a pulmonary capillary wedge pressure of >15 mmHg or a left ventricular end diastolic pressure of >18 mmHg. The incidence of AF was determined by review of electrocardiograms, ambulatory event monitor and inpatient telemetry recordings by using the standard definition [1, 12, 19]. Paroxysmal and persistent AF were included. Patients with prior MI, prior cardiac surgery, valvular disease, obstructive coronary artery disease, and HFpEF due to infiltrative disorders such as amyloidosis and genetic cardiomyopathies were excluded. The patients were not on anti-inflammatory or immunosuppressive medications.

Proteomics sample preparation

Blood samples were obtained in EDTA tubes in and processed immediately after collection. Plasma was separated and stored at -80°C for subsequent analysis. A 10 µl plasma/sample was depleted for 12 high abundance proteins using Pierce top 12 abundant protein depletion spin columns (Thermo) according to the manufacturer's recommendations. Proteins in the flow-through fractions were collected and processed further using proteomics sample preparation kit for plasma/serum (Biognosys). Proteins were digested using mass-spec grade trypsin (Promega) at a ratio of 50:1 (protein: enzyme) in NH_4HCO_3 buffer overnight at 37°C. The resulting peptides were purified using Pierce C18 tips (Thermo) and evaporated under vacuum.

Assessment of the association of plasma protein with AF, CMD, and HFpEF

Plasma samples from the patients were dived in study and control groups. Study and control

groups were matched based on age, sex, body-mass index, coexisting cardiovascular conditions and systemic diseases. Control group included samples from the patients with no AF or HFpEF or CMD. Study groups included patients with different concomitant diseases. The ACH group included with diagnosis of concomitant AF, CMD and HFpEF. The CH group included the patients with CMD and HFpEF, but no AF. We also studied the patients with HFpEF alone or CMD alone. LC-MS based untargeted and LFQ proteomic analysis was performed to investigate the dysregulated protein biomarkers in these patients' plasma samples. Circulating plasma proteins were screened in each group and compared with other groups to determine association of the proteins. Candidate biomarkers of their association were determined. Also, dysregulated proteins were identified for the shared mechanistic pathways in their coexistence.

LC-MS/MS analysis

MS analysis was performed on Orbitrap Elite™ Mass Spectrometer (Thermo Fisher Scientific) and coupled with a chromatographic nanoLC-ultra nanoflow system (Eksigent, Dublin, CA). Peptides were injected onto a trap column (150 µm ID × 3 cm in-house packed with ReproSil C18, 3 µm) coupled with an analytical column (75 µm ID × 10.5 cm, PicoChip column packed with ReproSil C18, 3 µm) (New Objectives, Inc., Woburn, MA) using a Dionex UltiMate 3000 Rapid Separation nanoLC (Thermo Fisher Scientific). A linear gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN) was used to separate the peptides over 120 minutes. Full MS scans were acquired from 400-2000 m/z at 60,000 resolving power using an isolation width of 1.0 m/z. The top fifteen most abundant precursor ions in each full MS scan were selected for MS/MS fragmentation by collision-induced dissociation (CID) at 35% normalized collision energy. Dynamic exclusion time was 58 seconds.

Data and statistical analysis

Raw MS data file analysis was performed with MaxQuant software version 1.6.0.16 (http://www.maxquant.org) [20], with a False Discovery Rate (FDR) ≤0.01 which was applied at the protein and peptide level. The searched parameters used in MS data processing were as follows: Oxidized Methionine (M), Acetylation (Protein N-term) as variable modifications and Carbamidomethyl (C) as fixed modifications with minimum 7 amino acids per peptide length. Furthermore, match between runs (MBR) was used as an advanced parameter with stringent 0.7 min as matching time window. LFQ was enabled in the global parameters to perform the quantitation analysis at MS/MS level. We also collected iBAQ to get the sampling depth of the overall sample set (Supplementary File 1). Peptides and corresponding protein identification were performed using the MaxQuant integrated Andromeda search engine (target-decoy approach with a reverse database). The data files were processed with a human consensus coding sequence (CCDS: ~35,000 sequences) database retrieved from Ensemble. A common contaminants list was appended to this FASTA file to facilitate their exclusion. For quality control analysis, MQ processed data was analyzed in RStudio [21]. The LFQ values were extracted from the protein group's files. Log2 transformed LFQ values corresponding to each identified protein were plotted for their correlation using Pearson correlation analysis. We also generated a box plot to show distribution of each sample quantitation values. Median quantile-based normalization was performed on the above dataset using limma package in RStudio (Boston, MA).

Results

Study samples

Plasma samples were collected from the patients during invasive coronary physiology study. The patients (median age 57-year-old, 83% female, 61% African Americans, mean LVEF 64±7%, mean body mass index 39±11 kg/m^2) were dived into study (n=13) and control (n=5) groups. Proteomics profile and dysregulated proteins in control and study groups including ACH group, CH group, HFpEF alone and CMD alone were detected and analyzed. We compared plasma proteomes of patients with AF, CMD and HFpEF with the control group to identify the dysregulated proteins as pathogenic markers of association between these three disease conditions.

Plasma proteomics profiling

The detailed proteomics workflow is described in **Figure 1A**. Plasma proteomic analysis covered a dynamic range of ~6 order of magnitude

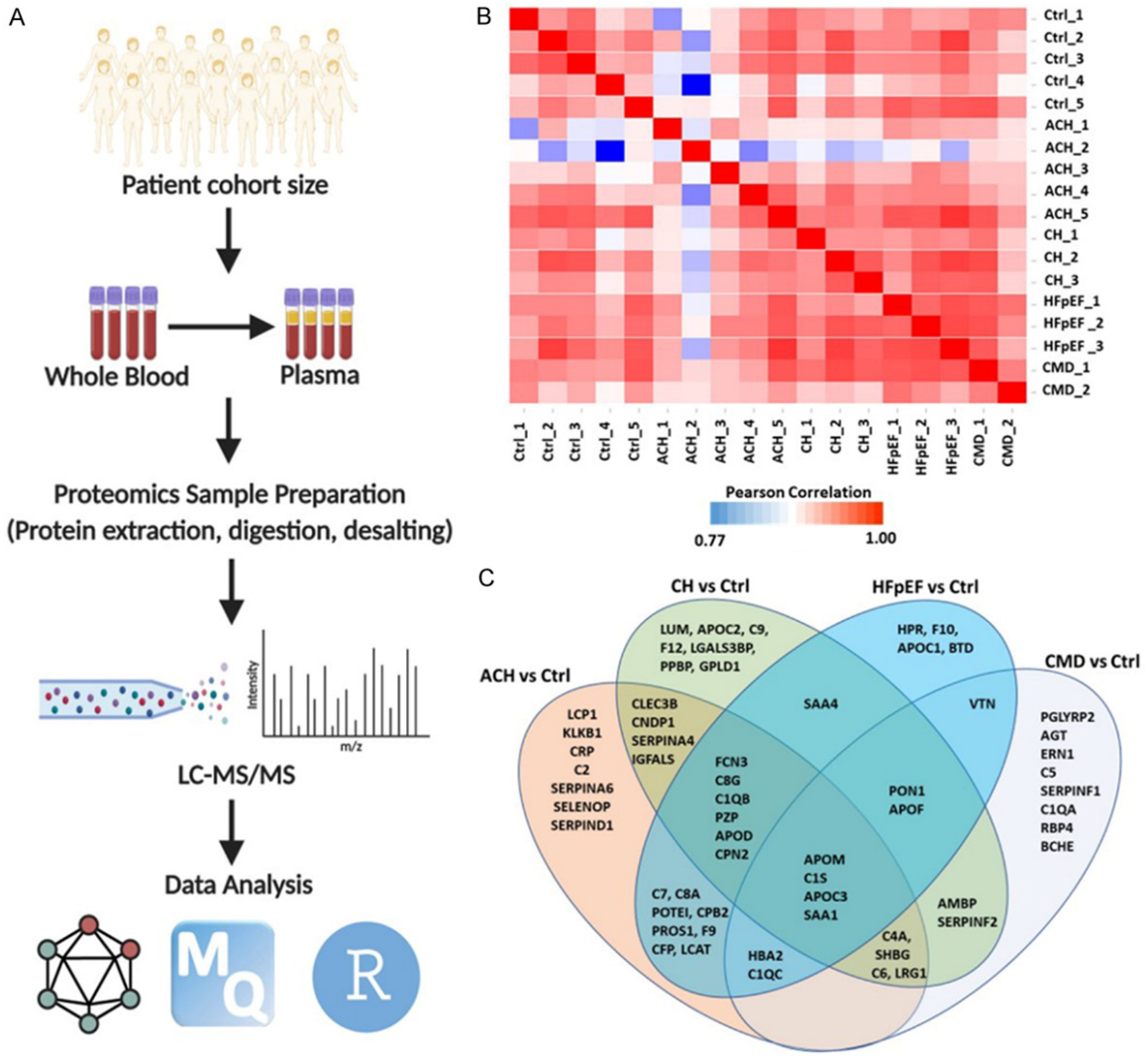

Figure 1. Plasma proteomics analysis. A. Experimental details depicting proteomics workflow; B. Pearson's correlation of independent biological replicates within and between the disease groups; C. Venn diagram showing identified differential proteins across patient versus control groups. Abbreviations: Ctrl, control group including patients' without atrial fibrillation (AF), coronary microvascular dysfunction (CMD), and heart failure with preserved ejection fraction (HFpEF); ACH, group including patients with concomitant AF, CMD, and HFpEF; CH, group including patients with concomitant CMD, and HFpEF; HFpEF, group of patients with HFpEF alone; CMD, group of patients with CMD alone.

(Supplementary Figure 1). Overall, we identified 253 proteins across the sample groups. The LFQ values were log2 transformed in RStudio for further analysis. The initial R based quality control analysis revealed a close distribution of the peptide's LFQ intensities per sample (Supplementary Figure 2), and correlation (Pearson correlation ~0.77-1.00) (**Figure 1B**). The data was normalized and fold change (FC) in each disease group compared to the control group was calculated. LFQ values for each protein were passed through a threshold and considered for further analysis if the signal was detected in at least 2 of the independent biological replicates. This resulted in 130 dysregulated proteins. The FC was calculated for each protein in the disease group with respect to the control group (Supplementary Table 1). Next, we applied a stringent threshold cutoff of FC ≥+1.5 and ≤-1.5, to filter the proteins between each disease group with respect to the control group (**Table 1**).

Table 1. Dysregulated plasma proteins

ACH		CH		HFpEF		CMD	
Protein ID	FC	Protein ID	FC	Protein ID	FC	Protein ID	FC
LCP1	-5.1	PON1	-4.1	PON1	-2.4	C1S	-5.5
KLKB1	-2.5	C1S	-3.6	SAA4	1.5	SHBG	-1.9
C4A	-2.4	SHBG	-1.9	POTEI	1.5	HBA2	-1.5
SHBG	-1.6	LUM	-1.6	PROS1	1.5	PGLYRP2	1.5
CLEC3B	1.5	CLEC3B	1.5	FCN3	1.6	C1QC	1.6
C6	1.5	SAA4	1.5	VTN	1.6	AMBP	1.6
CRP	1.5	SERPINA4	1.5	HPR	1.7	AGT	1.6
HBA2	1.5	AMBP	1.5	C8G	1.7	VTN	1.7
CNDP1	1.6	SERPINF2	1.6	APOM	1.7	APOM	1.7
C7	1.6	FCN3	1.7	C1QB	1.8	ERN1	1.8
FCN3	1.6	APOC2	1.7	C8A	1.8	C5	1.8
C2	1.6	C4A	1.7	HBA2	1.9	SERPINF1	1.8
SERPINA4	1.6	C9	1.7	C7	1.9	C6	2.1
IGFALS	1.7	CNDP1	1.7	F10	2.0	C4A	2.1
APOM	1.7	APOM	1.9	APOC1	2.0	C1QA	2.2
C8A	1.8	F12	2.0	C1QC	2.4	RBP4	2.3
POTEI	1.9	IGFALS	2.1	F9	2.5	SERPINF2	2.3
CPB2	1.9	C6	2.1	CFP	2.7	PON1	2.4
SERPINA6	2.1	LGALS3BP	2.4	LCAT	2.9	APOF	2.9
PROS1	2.3	C8G	2.4	APOF	2.9	BCHE	3.2
F9	2.5	C1QB	2.7	PZP	2.9	APOC3	5.0
SELENOP	2.6	CPN2	2.9	APOD	3.0	LRG1	23.8
C1QC	2.6	APOF	2.9	BTD	3.2	SAA1	105.5
CFP	2.7	PPBP	3.2	CPB2	3.6		
LCAT	2.9	APOD	3.9	C1S	3.6		
C1S	3.0	PZP	4.4	CPN2	4.2		
C8G	3.5	GPLD1	4.8	APOC3	13.1		
C1QB	3.5	APOC3	10.8	SAA1	81.4		
PZP	3.7	LRG1	38.8				
APOD	4.5	SAA1	66.8				
SERPIND1	6.3						
CPN2	7.5						
APOC3	11.3						
SAA1	13.0						
LRG1	16.9						

Abbreviations: ACH, indicates atrial fibrillation (AF), coronary microvascular disease (CMD) and heart failure with preserved ejection fraction (HFpEF); CH, CMD and HFpEF; ID, identifier; FC, fold change. Green color indices downregulated proteins and brown color upregulated proteins.

Dysregulated proteins

Among the 130 dysregulated proteins analyzed, 35 proteins passed the applied cut-off filter across different disease conditions (**Table 1**). The protein overlap analysis was performed to identify the common and unique proteins associated within each disease group with respect to the control (**Figure 1C**). STRING based protein network analysis revealed the involvement of dysregulated proteins in various physiological pathways such as inflammation, tissue remodeling, oxidative and metabolic stress, and complement and coagulation cascade (**Figure 2**). Across all disease groups, the top 3 reactome pathways were; 1) complement cascade (PROS1, CPB2, F2, C8A, C6, C8G, C4A, C7, C1R, C1S, C2, FCN3, CRP, C1QA, C1QB, C1QC, CPN2), 2) regulators of complement cascade (PROS1, CPB2, F2, C8A, C6, C8G, C4A, C7, C1R, C1S, C2, C1QA, C1QB, C1QC, CPN2), and 3) initial activation of complement system (C4A, CRP, C1R, CFP, C1S, C2, FCN3, C1QA, C1QB, C1QC). The complement cascade involves in inflammation, tissue remodeling, fibrosis, and other pathophysiological processes.

There were several upregulated proteins in patients with AF, CMD and HFpEF. FC in the levels of SAA1 (Serum Amyloid A1) was consistently elevated in all four study groups. SAA1 is a prominent acute phase protein. Another inflammatory marker, C-reactive protein (CRP), was also elevated in the ACH group with a 1.5-fold increase. FC of LRG1 (leucine-rich α-2-glycoprotein 1), a protein which has been shown to promote apoptosis and autophagy, demonstrated a significant increase in ACH (16.9), CH (38.8), CMD (23.8) groups. The levels of CPN2 were high in ACH, CH, and HFpEF groups. The levels of APOM, and APOC3 were

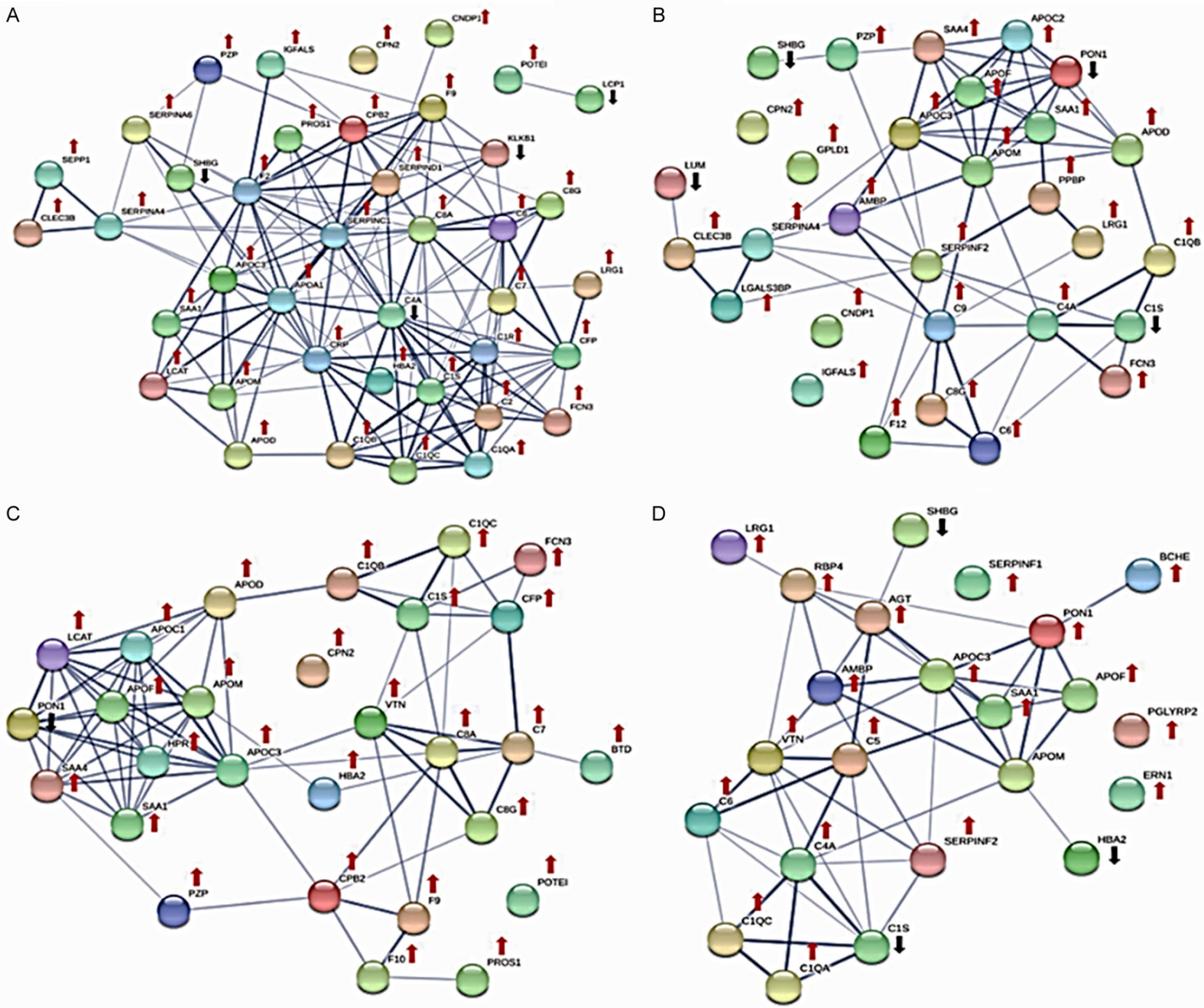
A
CNDP1
PZP
IGFALS
CPN2
POTEI
LCP1
F9
SERPINA6
PROS1
CPB2
KLKB1
SEPP1
SHBG
F2
SERPIND1
C8G
CLEC3B
SERPINA4
SERPINC1
C8A
C6
LRG1
APOC3
APOA1
C7
SAA1
C4A
CFP
CRP
C1R
LCAT
HBA2
APOM
C1S
C2
FCN3
APOD
C1QB
C1QC
C1QA
B
SHBG
PZP
SAA4
APOC2
PON1
APOF
CPN2
APOC3
SAA1
APOD
GPLD1
APOM
LUM
PPBP
AMBP
LRG1
CLEC3B
SERPINA4
C1QB
SERPINF2
LGALS3BP
C9
C4A
C1S
CNDP1
FCN3
IGFALS
C8G
F12
C6
C
C1QC
FCN3
C1QB
APOD
C1S
CFP
LCAT
APOC1
CPN2
APOM
APOF
VTN
PON1
C8A
C7
HPR
APOC3
BTD
HBA2
SAA4
SAA1
C8G
PZP
CPB2
F9
POTEI
F10
PROS1
D
LRG1
SHBG
RBP4
SERPINF1
BCHE
AGT
PON1
AMBP
APOC3
APOF
SAA1
PGLYRP2
VTN
C5
APOM
ERN1
C6
C4A
SERPINF2
HBA2
C1QC
C1S
C1QA

Figure 2. Protein association network. Network analysis of reported proteins associated with (A) ACH versus control, (B) CH versus control, (C) HFpEF versus control, and (D) CMD versus control, groups (Protein names correspond to gene IDs). Control group included patients' without atrial fibrillation (AF), coronary microvascular dysfunction (CMD), and heart failure with preserved ejection fraction (HFpEF). ACH group consisted of patients with concomitant AF, CMD, and HFpEF. CH group included patients with concomitant CMD, and HFpEF. Red arrow indicates upregulated proteins. Black arrow shows downregulated proteins.

increased. APOC3 showed more than a 10-fold increase in patients with ACH, CH, and HFpEF groups and 5-fold increase in CMD group when compared with normal group. APOC1, APOC2, and APOD were increased in ACH, CH, and HFpEF groups. Lastly, APOF was seen upregulated in CH, HFpEF and CMD groups. PPBP levels showed 3.2-fold increase in CH group. Coagulation factor F9 was increased in ACH, and HFpEF groups.

The proteins that were downregulated as reflected by a negative fold changes, are shown in **Table 1**. Complement 1S (C1S) was downregulated in patients with CMD and HFpEF in addition to patients with CMD alone. It displayed 3-fold increase in ACH group as well as HFpEF alone. C1S is part of immune response and coagulation. Reduced expression of Paraoxonase 1 (PON1) was also observed in ACH, CH, and HFpEF groups but it showed a 2.4-fold increase in CMD group. PON1 is a glycoprotein which has a role in inflammatory diseases. In the ACH group, lymphocyte cytosolic protein 1 (LCP1) was significantly downregulated (5-fold) when compared to control group. LCP1 has multiple functions critical for immunity and cellular processes. KLKB1, C4A, and SHBG were also low in ACH group. Reduced levels of LUM, and SHBG were observed across all the disease groups compared to normal individuals. Plasma LPA (APOA) levels were lower in CMD group. HBA2 was found to be lower in both CH and CMD groups. APOH levels were low in ACH group and APOA2 expression was lower in CH group. Several proteins exhibited similar levels of expression that have previously been implicated as potential biomarkers in various cardiovascular disease conditions. These proteins included GPX3, C9, HBB, VTN, GSN, SAA4, AGT, AMBP, TLN1, ATRN, C1RL, ITIH (1, 2, 3, 4) and apolipoproteins APOA1, APOA4, APOB, APOL1, and APOE, SERPIN (A1, A3, A5, A10, C1, F1, and G1).

Discussion

The main findings of our study include: 1) Discovery-based untargeted plasma proteomic analysis identified 35 proteins in association with AF, CMD and HFpEF. 2) SAA1, LRG1 and APOC3 were most consistently elevated markers of the coexistence of AF, CMD and HFpEF. 3) LCP1, PON1 and C1S were markedly downregulated in their associations. 4) Reduced levels of LCP1, KLKB1 and C4A were associated with AF in patients with CMD and HFpEF. 5) Combined downregulation of PON1 and C1S was a marker of the HFpEF and CMD. 6) Low PON1 was associated with HFpEF. 7) Low C1S was associated with CMD. These proteins are associated with the inflammatory processes, coagulation pathways, oxidative stress, metabolism, complement system and extracellular matrix remodeling. Thus, plasma proteomic profile may provide mechanistic insight into the association of AF, CMD and HFpEF and may serve as biomarkers.

Recently, we and others demonstrated that there are significant clinical associations between AF, CMD and HFpEF [8-12]. CMD is highly prevalent in patients with AF with or without HFpEF [12]. We found that CMD is a predictor of concomitant AF and HFpEF. CMD and AF or HFpEF are associated with higher risk of mortality and HF hospitalization. Therefore, it is critical to determine the patients who are at risk of developing concomitant AF, CMD and/or HFpEF. Our approach revealed several dysregulated proteins between the disease and control groups (**Figure 1C** and **Table 1**). Among 130 dysregulated proteins after quality control analysis (Supplementary Table 1), SAA1 was the most prominent biomarker for the association between AF, CMD and HFpEF, as depicted by highest FC across all groups. SAA1 is an acute phase protein and its level in plasma increases in response to inflammation or tissue injury [22, 23]. Elevated SAA may reflect an inflammatory state that promotes the occurrence and persistence of AF in CMD and HFpEF. SAA and CRP are primarily induced by the proinflammatory cytokine interleukin-6 (IL6) [23]. An elevated CRP level is known to predict the incidence of cardiovascular events [24]. A

1.5-fold increase was observed in CRP levels in ACH group. Also, an increase in the plasma levels of LRG1 was observed in ACH, CH, and CMD groups. LRG1 promotes apoptosis and autophagy through the TGFβ-smad1/5 signaling pathway by up-regulating ALK1, which exacerbates ischemia and reperfusion injury [25]. Our analysis shows that LRG1 is a biomarker of AF in CMD and HFpEF. Thus, elevated levels of SAA1 and LRG1 are valuable novel biomarkers to detect patients who are at the risk of developing concomitant AF, CMD and HFpEF.

Furthermore, we found several downregulated proteins in coexistence of AF, CMD and HFpEF or in individual disease condition. Among those proteins, LCP1, PON1 and C1S were more noticeable. C1S levels were significantly low in CH group, and in patients with CMD. Down regulation of C1S is a promising biomarker for assessing the risk of CMD since C1S was particularly lower in patients with CMD alone. Decreased C1 complex activity has been associated with impaired immune response which is part of the inflammatory process [26]. However, C1S was upregulated (3-fold increase) in ACH, and HFpEF groups. LCP1 was downregulated by 5-fold in the ACH group. LCP1 is critical for immunity and cellular processes. The levels of CPN2 were high in ACH, and HFpEF groups. CPN2 inhibits fibrinolysis and may be up-regulated in myocardial infarction during clot formation over a ruptured plaque in an attempt to limit complement activation and restrict inflammation [27]. The complement system is an essential component of immune response and regulates inflammatory processes [26-29]. Downregulation of PON1 in CH, and HFpEF groups is significant as a likely marker for the association of these disease processes. PON1 is a glycoprotein which has a role in inflammatory diseases and acts as anti-atherosclerotic component of HDL [30]. Thus, the measurement of the levels of LCP1, PON1 and C1S in plasma samples can guide clinicians for patient's care by determining the susceptibility to CMD, HFpEF and AF.

Overall, our study presents valuable biomarkers for risk stratification and mechanistic insight into the association between AF, CMD and HFpEF. This has significant clinical implications. By studying human plasma samples, our findings indicate that circulating biomarkers such as SAA1, LRG1, APOC3, LCP1, PON1 and C1S, have high translational potential to predict the association of AF, CMD, and HFpEF. Detecting high-risk patients with these biomarkers will provide an opportunity to initiate preventive measures and close monitoring of the patient for early diagnosis and management of the CMD, AF and HFpEF. Ultimately, this may help to reduce morbidity and mortality in those patients. These markers need to be tested in larger cohort.

Proteomic profiling of our study population demonstrated that the association of AF, CMD and HFpEF involves multiple physiological pathways including inflammation, complement system, coagulation cascade, oxidative stress, metabolism, fibrosis, and extracellular remodeling. Alteration of intracellular and extracellular matrix protein complexes in the plasma indicates tissue remodeling in these disease processes. Functional and structural myocardial remodeling in atria and ventricle is essential in the coexistence of AF, HFpEF and CMD [1-3, 17-19, 31-33]. Myocardial tissue remodeling can be triggered by multiple clinical or molecular factors, and includes fibrosis, myocyte loss, inflammation, stretch, disrupted electrical conduction, cardiomyocyte and extracellular matrix remodeling [23, 24, 28]. Recently, we demonstrated mitochondrial dysfunction, impaired energetics and oxidative stress in mouse and human atrial myocardium in association with AF and HF [17]. CMD with abnormal microcirculation attenuates coronary flow augmentation in response to stress and it leads to demand-supply mismatch, and myocardial ischemia [6]. CMD is found to be associated with myocardial fibrosis and inflammation along with ischemia [6, 11, 33, 34]. These pathways in atrial and ventricular myocardial remodeling contribute to the pathophysiological association between AF, CMD and HFpEF. Novel mechanistic insights into their relationship will facilitate the development of new therapeutic approaches for the primary and secondary prevention of AF, CMD and HFpEF.

There are limitations in our study including sample size. However, this is a discovery-based untargeted plasma proteomic analysis with the independent patient replicates and therefore there is no prerequisite of larger number of samples. Current samples are sufficient to ma-

ke scientific conclusions in these analysis. Our findings provide biomarkers for the association between AF and other disease conditions. These novel biomarkers are required to be tested in future targeted validation studies in larger cohorts. There are missing LFQ values of some proteins across the independent replicates. To overcome this limitation, we considered the proteins as biomarker only if they were detected in at least two of the independent patient samples in each group.

In conclusion, this plasma proteomic analysis has identified mechanistic pathways and novel biomarkers linking AF, CMD and HFpEF. Our study demonstrates translational potential with the discovery of circulating biomarkers such as SAA1, LRG1, APOC3, LCP1, PON1 and C1S to predict the association of AF, CMD, and HFpEF. These markers show the role of oxidative/metabolic stress, inflammation, extracellular remodeling, and coagulation pathways in shared mechanisms of these coexisting disease processes. Thus, these circulating markers may lay the groundwork for future studies in detecting underlying pathogenesis and the patient population at risk for the association between AF, CMD and HFpEF.

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Disclosure of conflict of interest

None.

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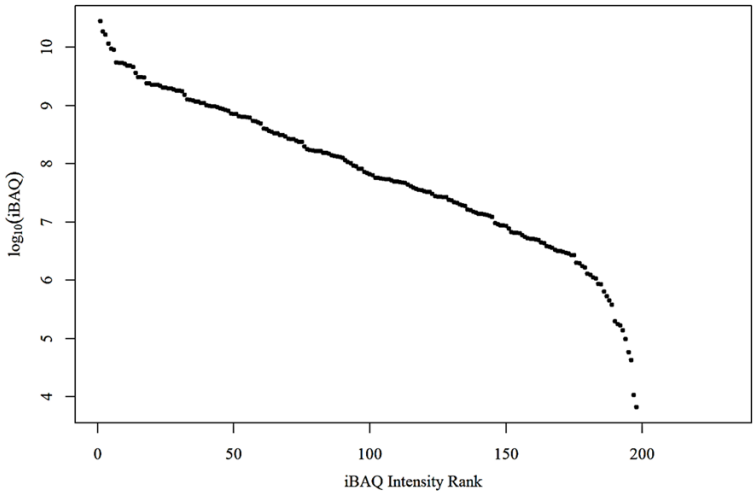
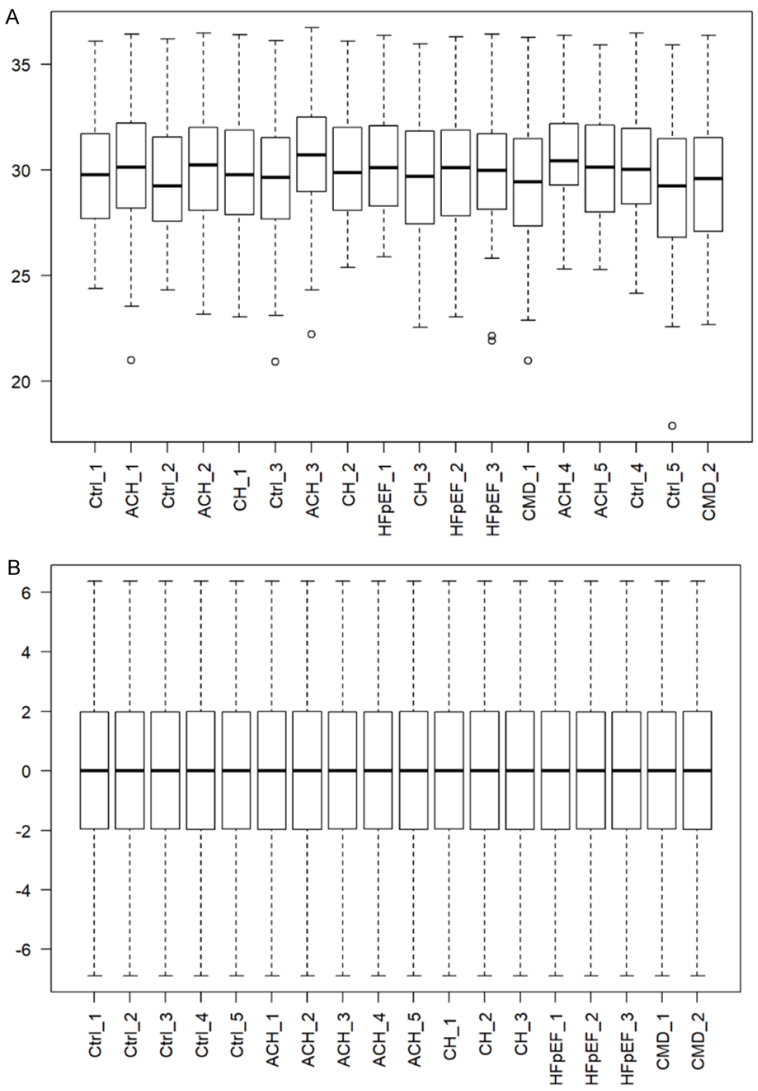
Supplementary method

Proteome MS data quality control (QC)

Eighteen plasma samples from CVD patients [disease groups: atrial fibrillation (AF) + Coronary Microvascular Disease (CMD) + Heart Failure with Preserved Ejection Fraction (HFpEF), CMD, and HFpEF along with control samples] were collected at the University of Chicago Medical Center. The most abundant proteins were depleted using the commercial kit (please refer to method section of the manuscript). We digested the individual samples utilizing a bottom-up proteomics sample prep work flow (experimental details are available in the method section of the manuscript). Post sample prep, the samples were run with nano-LC-MS/MS and collected data was searched with MaxQuant for label free quantification (LFQ). Here, we analyzed the overall proteome coverage in the samples. For protein analysis, we used LFQ intensity (LFQ: Label-free quantification). These intensities are based on the (raw) intensities and normalized on multiple levels to make sure that profiles of LFQ intensities across samples accurately reflect the relative amounts of the proteins and iBAQ (Intensity Based Absolute Quantification: iBAQ values calculated by MaxQuant are the (raw) intensities divided by the number of theoretical peptides. iBAQ values are proportional to the molar quantities of the proteins). Overall, we identified ~200 proteins. The quant data is presented below.



Supplementary Figure 1. Proteomic sampling depth. Running protein count by iBAQ revealed a sampling depth of ~6 orders of magnitude.



Supplementary Figure 2. Box plot. Before median quantile normalization (A). Median quantile normalized data (B).

Supplementary Table 1. Dysregulated Plasma Proteins

Gene ID	HGNC ID	Protein names	ACH	CH	HFpEF	CMD
C1S	HGNC:1247	complement C1s	3.0	-3.6	3.6	-5.5
SHBG	HGNC:10839	sex hormone binding globulin	-1.6	-1.9	1.0	-1.9
HBA2	HGNC:4824	hemoglobin subunit alpha 2	1.5	-0.7	1.9	-1.5
LUM	HGNC:6724	lumican	0.2	-1.6	-0.1	-0.8
LPA	HGNC:6667	lipoprotein(a)	1.1	1.1	0.7	-0.5
CPN2	HGNC:2313	carboxypeptidase N subunit 2	7.5	2.9	4.2	-0.2
IGFALS	HGNC:5468	insulin like growth factor binding protein acid labile subunit	1.7	2.1	0.8	-0.1
C8G	HGNC:1354	complement C8 gamma chain	3.5	2.4	1.7	-0.1
SERPINA6	HGNC:1540	serpin family A member 6	2.1	1.1	1.4	0.1
C7	HGNC:1346	complement C7	1.6	0.7	1.9	0.2
SERPIND1	HGNC:4838	serpin family D member 1	6.3	0.3	-0.2	0.2
C2	HGNC:1248	complement C2	1.6	1.2	1.2	0.2
APOE	HGNC:613	apolipoprotein E	0.3	0.8	0.1	0.3
HP	HGNC:5141	haptoglobin	0.5	0.9	0.8	0.3
FCN3	HGNC:3625	ficolin 3	1.6	1.7	1.6	0.3
TLN1	HGNC:11845	talin 1	0.8	1.1	0.7	0.4
PZP	HGNC:9750	PZP alpha-2-macroglobulin like	3.7	4.4	2.9	0.4
ITIH3	HGNC:6168	inter-alpha-trypsin inhibitor heavy chain 3	0.9	0.3	0.9	0.4
F12	HGNC:3530	coagulation factor XII	1.4	2.0	1.1	0.5
FN1	HGNC:3778	fibronectin 1	1.3	0.9	1.1	0.5
HBB	HGNC:4827	hemoglobin subunit beta	0.3	0.4	0.8	0.5
CLEC3B	HGNC:11891	C-type lectin domain family 3 member B	1.5	1.5	0.9	0.6
C1QB	HGNC:1242	complement C1q B chain	3.5	2.7	1.8	0.6
C8B	HGNC:1353	complement C8 beta chain	1.0	1.0	0.8	0.6
CD14	HGNC:1628	CD14 molecule	0.9	0.8	0.8	0.6
SERPINA7	HGNC:11583	serpin family A member 7	0.9	0.9	1.0	0.6
APOH	HGNC:616	apolipoprotein H	-0.9	1.2	0.9	0.6
F11	HGNC:3529	coagulation factor XI	0.9	1.2	0.9	0.7
ORM2	HGNC:8499	orosomucoid 2	0.6	0.5	0.5	0.7
VCL	HGNC:12665	vinculin	0.8	1.0	0.8	0.7
GPLD1	HGNC:4459	glycosylphosphatidylinositol specific phospholipase D1	0.6	4.8	0.6	0.7
QSOX1	HGNC:9756	quiescin sulfhydryl oxidase 1	1.0	0.9	1.0	0.7
ECM1	HGNC:3153	extracellular matrix protein 1	1.1	1.1	0.8	0.7
CNDP1	HGNC:20675	carnosine dipeptidase 1	1.6	1.7	1.1	0.7
CFD	HGNC:2771	complement factor D	0.8	0.8	0.8	0.8
F10	HGNC:3528	coagulation factor X	1.3	0.9	2.0	0.8
SERPINA4	HGNC:8948	serpin family A member 4	1.6	1.5	1.4	0.8
IGLL5	HGNC:38476	immunoglobulin lambda like polypeptide 5	-0.4	0.2	0.2	0.8
CPN1	HGNC:2312	carboxypeptidase N subunit 1	1.1	0.9	1.0	0.8
C4BPA	HGNC:1325	complement component 4 binding protein alpha	0.6	1.2	0.9	0.8
C4BPB	HGNC:1328	complement component 4 binding protein beta	0.8	0.8	0.7	0.8
FETUB	HGNC:3658	fetuin B	1.0	1.1	0.9	0.8
AHSG	HGNC:349	alpha 2-HS glycoprotein	0.2	0.7	0.7	0.8
SERPINA10	HGNC:15996	serpin family A member 10	1.0	0.7	1.0	0.8
GPX3	HGNC:4555	glutathione peroxidase 3	1.3	1.1	1.3	0.8
APOL1	HGNC:618	apolipoprotein L1	1.2	1.3	1.1	0.8
BTD	HGNC:1122	biotinidase	1.1	1.0	3.2	0.9
ATRN	HGNC:885	attractin	1.3	1.0	1.3	0.9
C9	HGNC:1358	complement C9	1.3	1.7	1.2	0.9
LCAT	HGNC:6522	lecithin-cholesterol acyltransferase	2.9	1.2	2.9	0.9

C1RL	HGNC:21265	complement C1r subcomponent like	1.0	1.0	1.4	0.9
CPB2	HGNC:2300	carboxypeptidase B2	1.9	1.3	3.6	0.9
ORM1	HGNC:8498	orosomucoid 1	0.9	1.0	0.9	0.9
APOC2	HGNC:609	apolipoprotein C2	1.3	1.7	1.4	0.9
APOC1	HGNC:607	apolipoprotein C1	1.3	1.5	2.0	0.9
APOD	HGNC:612	apolipoprotein D	4.5	3.9	3.0	0.9
APOA4	HGNC:602	apolipoprotein A4	0.8	0.9	0.9	0.9
ITIH4	HGNC:6169	inter-alpha-trypsin inhibitor heavy chain 4	1.0	1.0	1.0	0.9
LBP	HGNC:6517	lipopolysaccharide binding protein	1.1	1.2	0.9	1.0
APOA1	HGNC:600	apolipoprotein A1	1.0	0.9	1.0	1.0
APCS	HGNC:584	amyloid P component, serum	1.1	0.8	0.9	1.0
C8A	HGNC:1352	complement C8 alpha chain	1.8	0.4	1.8	1.0
C3	HGNC:1318	complement C3	1.0	1.0	1.0	1.0
FGA	HGNC:3661	fibrinogen alpha chain	0.8	0.9	1.0	1.0
CFP	HGNC:8864	complement factor properdin	2.7	1.3	2.7	1.0
FGG	HGNC:3694	fibrinogen gamma chain	1.0	1.1	1.0	1.0
SERPINA5	HGNC:8723	serpin family A member 5	1.1	1.1	1.1	1.0
LCP1	HGNC:6528	lymphocyte cytosolic protein 1	-5.1	1.0	1.0	1.0
HPX	HGNC:5171	hemopexin	0.9	1.0	1.0	1.0
C4B	HGNC:1324	complement C4B (Chido blood group)	1.0	1.0	1.0	1.0
FGB	HGNC:3662	fibrinogen beta chain	0.8	1.0	1.0	1.0
PRG4	HGNC:9364	proteoglycan 4	0.9	1.2	1.4	1.0
F9	HGNC:3551	coagulation factor IX	2.5	1.1	2.5	1.0
KNG1	HGNC:6383	kininogen 1	0.9	1.0	1.0	1.0
SERPINA1	HGNC:8941	serpin family A member 1	1.1	1.1	1.0	1.0
SERPINC1	HGNC:775	serpin family C member 1	0.8	0.8	0.8	1.1
APOB	HGNC:603	apolipoprotein B	0.9	0.9	0.9	1.1
CP	HGNC:2295	ceruloplasmin	1.0	1.0	1.0	1.1
ITIH1	HGNC:6166	inter-alpha-trypsin inhibitor heavy chain 1	1.0	0.8	0.9	1.1
POTEI	HGNC:37093	POTE ankyrin domain family member I	1.9	1.2	1.5	1.1
LGALS3BP	HGNC:6564	galectin 3 binding protein	1.1	2.4	1.4	1.1
B2M	HGNC:914	beta-2-microglobulin	0.7	0.7	0.5	1.1
F2	HGNC:3535	coagulation factor II, thrombin	0.8	1.0	1.1	1.1
GC	HGNC:4187	GC vitamin D binding protein	1.0	1.0	1.0	1.1
AZGP1	HGNC:910	alpha-2-glycoprotein 1, zinc-binding	1.1	1.0	0.9	1.1
CFH	HGNC:4883	complement factor H	0.7	0.5	0.8	1.1
PPBP	HGNC:9240	pro-platelet basic protein	1.0	3.2	1.2	1.1
SERPING1	HGNC:1228	serpin family G member 1	1.1	1.1	1.0	1.1
SERPINA3	HGNC:16	serpin family A member 3	1.0	1.1	1.0	1.1
TF	HGNC:11740	transferrin	0.7	0.2	0.9	1.1
TTR	HGNC:12405	transthyretin	1.0	0.9	1.2	1.1
ITIH2	HGNC:6167	inter-alpha-trypsin inhibitor heavy chain 2	1.0	1.0	1.1	1.1
HPR	HGNC:5156	haptoglobin-related protein	1.4	0.9	1.7	1.1
SELENOP	HGNC:10751	selenoprotein P	2.6	1.3	1.0	1.2
A2M	HGNC:7	alpha-2-macroglobulin	1.0	1.2	0.8	1.2
A1BG	HGNC:5	alpha-1-B glycoprotein	1.1	1.0	1.0	1.2
PLG	HGNC:9071	plasminogen	0.5	0.9	1.0	1.2
VWF	HGNC:12726	von Willebrand factor	0.9	1.2	0.9	1.2
HRG	HGNC:5181	histidine rich glycoprotein	1.1	0.8	1.1	1.2
AFM	HGNC:316	afamin	0.9	0.7	1.1	1.2
APOA2	HGNC:601	apolipoprotein A2	0.1	-0.7	0.5	1.2
CFB	HGNC:1037	complement factor B	1.0	1.1	1.2	1.2

CLU	HGNC:2095	clusterin	0.9	0.9	1.0	1.2
PROS1	HGNC:9456	protein S	2.3	1.4	1.5	1.3
KLKB1	HGNC:6371	kallikrein B1	-2.5	-0.2	-0.7	1.3
C1R	HGNC:1246	complement C1r	0.3	0.4	0.0	1.3
CFI	HGNC:5394	complement factor I	-0.5	0.4	0.7	1.4
SAA4	HGNC:10516	serum amyloid A4, constitutive	1.3	1.5	1.5	1.4
GSN	HGNC:4620	gelsolin	1.0	1.0	1.3	1.4
CRP	HGNC:2367	C-reactive protein	1.5	1.3	1.1	1.4
PGLYRP2	HGNC:30013	peptidoglycan recognition protein 2	1.1	1.2	1.0	1.5
C1QC	HGNC:1245	complement C1q C chain	2.6	1.0	2.4	1.6
AMBP	HGNC:453	alpha-1-microglobulin/bikunin precursor	1.1	1.5	1.3	1.6
AGT	HGNC:333	angiotensinogen	0.8	1.0	1.0	1.6
VTN	HGNC:12724	vitronectin	0.9	0.9	1.6	1.7
APOM	HGNC:13916	apolipoprotein M	1.7	1.9	1.7	1.7
ERN1	HGNC:3449	endoplasmic reticulum to nucleus signaling 1	1.3	1.2	1.1	1.8
C5	HGNC:1331	complement C5	1.4	0.7	1.3	1.8
SERPINF1	HGNC:8824	serpin family F member 1	0.8	0.3	0.5	1.8
C6	HGNC:1339	complement C6	1.5	2.1	1.1	2.1
C4A	HGNC:1323	complement C4A (Rodgers blood group)	-2.4	1.7	-0.2	2.1
C1QA	HGNC:1241	complement C1q A chain	1.3	1.3	1.3	2.2
RBP4	HGNC:9922	retinol binding protein 4	-1.0	-0.5	0.9	2.3
SERPINF2	HGNC:9075	serpin family F member 2	1.3	1.6	1.4	2.3
PON1	HGNC:9204	paraoxonase 1	-0.2	-4.1	-2.4	2.4
APOF	HGNC:615	apolipoprotein F	1.0	2.9	2.9	2.9
BCHE	HGNC:983	butyrylcholinesterase	1.0	0.9	1.0	3.2
APOC3	HGNC:610	apolipoprotein C3	11.3	10.8	13.1	5.0
LRG1	HGNC:29480	leucine rich alpha-2-glycoprotein 1	16.9	38.8	1.0	23.8
SAA1	HGNC:10513	serum amyloid A1	13.0	66.8	81.4	105.5

Note: Fold change values (cut-off set to +/- 1.5) reflects differentially expressed proteins across patient *versus* control groups. Abbreviations: ACH indicates atrial fibrillation/coronary microvascular disease/heart failure with preserved ejection fraction; CH, coronary microvascular disease/heart failure with preserved ejection fraction; CMD, coronary microvascular disease; ID, identifier; FC, fold change. *Green* color indices downregulated proteins.