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ORIGINAL RESEARCH

Proteomics and Precise Exercise Phenotypes in Heart Failure With Preserved Ejection Fraction: A Pilot Study

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BACKGROUND: While exercise impairments are central to symptoms and diagnosis of heart failure with preserved ejection fraction (HFpEF), prior studies of HFpEF biomarkers have mostly focused on resting phenotypes. We combined precise exercise phenotypes with cardiovascular proteomics to identify protein signatures of HFpEF exercise responses and new potential therapeutic targets.

METHODS AND RESULTS: We analyzed 277 proteins (Olink) in 151 individuals (N=103 HFpEF, 48 controls; 62±11 years; 56% women) with cardiopulmonary exercise testing with invasive monitoring. Using ridge regression adjusted for age/sex, we defined proteomic signatures of 5 physiological variables involved in HFpEF: peak oxygen uptake, peak cardiac output, pulmonary capillary wedge pressure/cardiac output slope, peak pulmonary vascular resistance, and peak peripheral O₂ extraction. Multiprotein signatures of each of the exercise phenotypes captured a significant proportion of variance in respective exercise phenotypes. Interrogating the importance (ridge coefficient magnitude) of specific proteins in each signature highlighted proteins with putative links to HFpEF pathophysiology (eg, inflammatory, profibrotic proteins), and novel proteins linked to distinct physiologies (eg, proteins involved in multiorgan [kidney, liver, muscle, adipose] health) were implicated in impaired O₂ extraction. In a separate sample (N=522, 261 HF events), proteomic signatures of peak oxygen uptake and pulmonary capillary wedge pressure/cardiac output slope were associated with incident HFpEF (odds ratios, 0.67 [95% CI, 0.50–0.90] and 1.43 [95% CI, 1.11–1.85], respectively) with adjustment for clinical factors and B-type natriuretic peptides.

CONCLUSIONS: The cardiovascular proteome is associated with precision exercise phenotypes in HFpEF, suggesting novel mechanistic targets and potential methods for risk stratification to prevent HFpEF early in its pathogenesis.

Key Words: biomarkers ■ exercise ■ hemodynamics ■ HFpEF ■ proteomics

Heat failure (HF) with preserved ejection fraction (HFpEF) accounts for about half of HF cases, with a rising prevalence.¹ Despite recent therapeutic successes,² prevention, diagnosis, and prognosis

assessment in HFpEF remain limited by its heterogeneous clinical presentation and varied pathophysiological origins.³ Given the importance of exertional intolerance as a cardinal manifestation of HFpEF whether or not

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CLINICAL PERSPECTIVE

What Is New?

- Heart failure with preserved ejection fraction (HFpEF) is a heterogeneous condition with limited treatment options, and there is an unmet need to identify biomarkers and mechanisms of increased risk earlier in its development.
- Exercise assessments are uniquely able to characterize physiological contributors to HFpEF and to guide molecular discovery.
- Aligning precision exercise phenotypes with proteomic profiling in HFpEF identified novel risk markers of specific physiological abnormalities relevant to HFpEF development.

What Are the Clinical Implications?

- Multiprotein signatures of physiological exercise response patterns identified distinct molecular profiles of cardiac performance and peripheral skeletal muscle oxygen use in HFpEF with implications for future HF development and potential therapeutic targets.
- Multiprotein scores defined by 2 integrative physiological measures—peak oxygen uptake and pulmonary capillary wedge pressure/cardiac output slope—were associated with long-term HFpEF development in a separate community-based sample.

Nonstandard Abbreviations and Acronyms

CPET	cardiopulmonary exercise testing
CO	cardiac output
FHS	Framingham Heart Study
HFpEF	heart failure with preserved ejection fraction
HFREF	heart failure with reduced ejection fraction
VO₂	oxygen uptake

resting congestion exists, the use of exercise testing to quantify physiological abnormalities underlying these symptoms is an important component of HFpEF care and investigation.^{4,5} Physiological exercise response patterns can be used to define specific impairments across various organ systems in HFpEF, including decreased cardiorespiratory fitness (peak oxygen uptake [VO₂]), impaired peripheral oxygen extraction,⁶ abnormalities in pulmonary vascular–right ventricular coupling during exercise,⁷ and left ventricular–vascular interactions that result in a steep rise in left heart filling pressures.^{8,9} Prior

investigations of these exercise phenotypes in HFpEF primarily focused on diagnosis and assessing phenotypes, disease severity, and therapeutic response.^{5,10} While recent advances in molecular phenotyping of tissues and biofluids in HF provide a lens into potential mechanisms and functional biomarkers of disease,^{11–13} few studies have used these tools in combination with detailed phenotyping during exercise. Uniting exercise and molecular phenotypes provides an opportunity not only to define potential mechanisms of HFpEF but also to noninvasively interrogate an individual's propensity to develop HFpEF without the need for invasive measurements during exercise.

Here, we defined the relation of a broad resting cardiovascular proteome (277 proteins) with key physiological exercise response patterns in 151 individuals with and without HFpEF. Participants were drawn from a cohort of individuals referred for cardiopulmonary exercise testing (CPET) with invasive hemodynamic monitoring for evaluation of dyspnea with exertion and therefore largely represent individuals with HFpEF without overt congestion at rest. We employed statistical (machine) learning approaches to define proteomic signatures of hemodynamic and functional measures, using these models to prioritize contributions of known/novel pathways to divergent HFpEF pathophenotypes. Given the relevance of proteomics to early disease detection, we evaluated whether these signatures identify individuals at high risk for incident HFpEF in a distinct and carefully phenotyped at-risk, community-based cohort. Our overall objective was to assess whether cardiovascular proteomics related to individual-level physiological performance during exercise would identify functional pathways and clinical HFpEF risk (Figure 1).

METHODS

Data Sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request. Anonymized data and materials from the Framingham Heart Study (FHS) have been made publicly available through the National Institutes of Health Database of Genotypes and Phenotypes, and procedures for obtaining FHS data can be accessed at www.framinghamheartstudy.org.

Study Samples

Hemodynamic Cardiopulmonary Exercise Testing

We investigated individuals referred for CPET with invasive hemodynamic monitoring for dyspnea evaluation from 2006 to 2018. Eligible participants were >40 years of age, with blood samples, ejection fraction

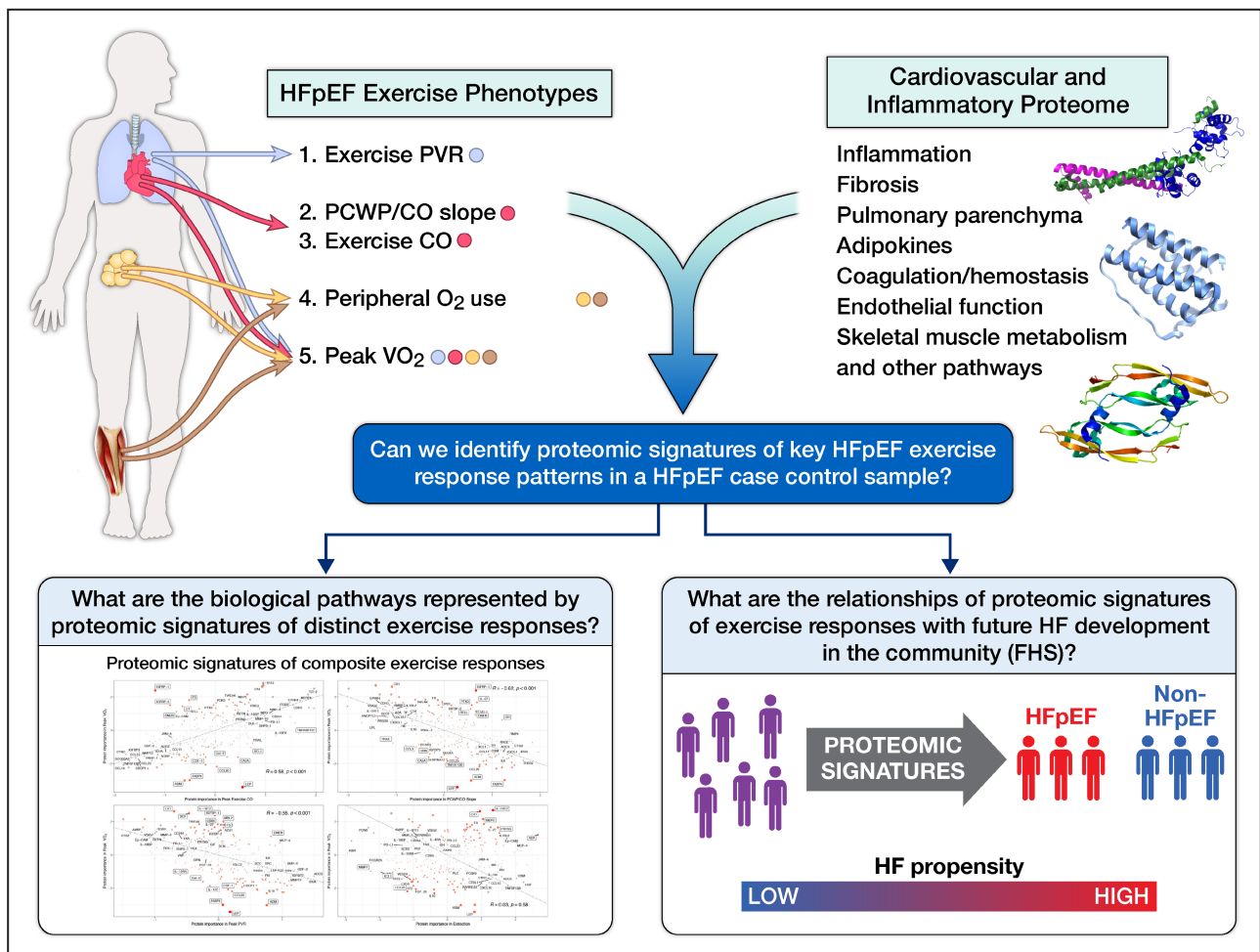


Figure 1. Overview of the study design.

CO indicates cardiac output; FHS, Framingham Heart Study; HFpEF, heart failure with preserved ejection fraction; O₂, oxygen; PCWP/CO, slope of the pulmonary capillary wedge pressure to cardiac output ratio; PVR, pulmonary vascular resistance; and VO₂, oxygen uptake.

(EF) ≥50%, and without known disease processes outside of HFpEF affecting exercise performance (eg, congenital heart disease, moderate or greater valvular heart disease, known infiltrative cardiomyopathy, oxygen-dependent lung disease, lung transplant evaluation or prior lung transplant, mitochondrial disease, postural orthostatic tachycardia syndrome). From this sample, we used a case–control design including 119 participants meeting a hemodynamic diagnosis of HFpEF, as defined by excessive pulmonary capillary wedge pressure (PCWP) response to exercise (PCWP/cardiac output [CO] slope ≥2 mmHg/L per min⁸). We also selected 57 controls with normal PCWP (PCWP/CO <2 mmHg/L per min) and pulmonary arterial pressure response to exercise (mean pulmonary artery pressure/CO slope <3 mmHg/L per min⁷) and with preserved cardiorespiratory fitness (peak VO₂ ≥80% predicted¹⁴). After excluding individuals with missing values for phenotypes, covariates, or proteomics, our final analytic sample size included 151 individuals

(N=103 with HFpEF and N=48 controls). All participants provided written informed consent. Study protocols were approved by the Mass General Brigham and Boston University Medical Campus Institutional Review Boards.

Association With Future HFpEF

We evaluated the association of multiprotein scores developed in our hemodynamic CPET sample with incident HF in FHS participants with long-term follow-up and adjudication of HF subtype (HFpEF versus HF with reduced EF [HFrEF]¹⁵). FHS offspring participants were selected in a nested case–control design, with cases defined as individuals who developed incident HF between the seventh examination cycle and December 31, 2016, and controls matched 1:1 on sex and <5 year difference in birth year (N=261 in each group). HF events were adjudicated on the basis of standardized protocols and were classified as HF with preserved

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(EF >50%), reduced (EF ≤50%), or unknown EF, based on assessment within 1 year before the onset of HF as long as no myocardial infarction occurred in the interim.¹⁵

Cardiopulmonary Exercise Testing

In our hemodynamic CPET sample, maximum effort CPET with pulmonary and radial arterial (when technically feasible) catheters was performed on a cycle ergometer with an incremental ramp protocol. Peak VO_2 was assessed as the maximum 30-second value during exercise. CO was calculated using the Fick method, and PCWP and mean pulmonary artery pressures were measured at end expiration with values recorded during each minute of exercise. Other hemodynamic measures were obtained during each minute of loaded exercise, and the slope of the relation of the PCWP versus CO was calculated via least squares regression.⁹

Protein Quantification

Stored plasma samples from the superior vena cava at rest and peak exercise were quantified in participants from the CPET sample via proximity extension assay methods (Olink, Uppsala, Sweden; Cardiovascular-II, Cardiovascular-III, Inflammation, Cardiometabolic panels). These panels were chosen due to presence of known and novel cardiovascular biomarkers and inflammatory proteins, given their putative links to HFpEF physiology.^{13,16} From the initial set of proteins and samples, we filtered out 81 proteins with ≥50% values below the reported limit of detection or unreported. All other proteins' levels were analyzed without imputation. Proteins measured in >1 Olink panel (10) had the value that was reported for the Cardiovascular-II/Cardiovascular-III panel included; the relations between proteins repeated across panels was high ($r > 0.86$ – 0.99). Our final analytic sample included 277 proteins (Table S1). Given our hypothesis that the rest proteome could identify important pathways relevant to HFpEF, we included only the rest proteome in analyses. From these, we excluded BNP (B-type natriuretic peptide) and NT-proBNP (N-terminal pro-B-type natriuretic peptide) as eligible proteins for regression, given their established roles in HF prediction. We included all 275 remaining proteins for analysis (without additional filtering for variability given that this was a discovery effort). Protein quantification in the FHS was performed using similar laboratory methods on stored plasma samples using the same 4 Olink panels.

Statistical Analysis

We first developed multiprotein *signatures* for each of 5 key physiological domains characterizing responses to exercise in HFpEF⁴: global cardiorespiratory fitness (peak VO_2), increased left heart filling pressures (PCWP/CO slope), cardiac output reserve (peak CO), peak pulmonary vascular resistance (PVR), and defect

in peripheral O_2 extraction as assessed by the arteriovenous oxygen difference. Given the greater number of proteins relative to the number of participants in our analysis, we used ridge regression models, in which each exercise phenotype was the dependent variable (log-transformed as appropriate and standardized), and the assayed proteins (penalized, standardized) were independent variables. Age and sex were forced into each model (not penalized, not standardized). Ridge regression was selected (as opposed to other types of penalized regression) to allow each protein to contribute independently to the model (adjusted for other proteins, age, and sex) to facilitate the broadest inclusion of proteins in the discovery effort and to account for collinearity across proteins. In this approach, the ridge regression coefficient for each protein can be treated as a quantitative metric of the “importance” of that protein to a given phenotype (its contribution to the variance explained in the phenotype).

We next sought to identify potential distinct and shared pathways of each of the hemodynamic profiles in exploratory analyses. We examined the relation of each protein's ridge coefficient for a given phenotype with its ridge coefficient for peak VO_2 (an integrative measure of fitness). The rationale for this approach was that proteins with similar importance to peak VO_2 (ridge β_{VO_2}) and a given hemodynamic phenotype (ridge $\beta_{\text{hemodynamic}}$) would identify shared pathways of exertional limitation and hemodynamic dysfunction applicable to HFpEF (ridge $\beta_{\text{VO}_2} = \text{ridge } \beta_{\text{hemodynamic}}$). Proteins with discordant relations between phenotype and peak VO_2 would suggest that a protein's association with fitness may not be as closely explained by its relation with a given hemodynamic contributor to peak VO_2 . We calculated a standardized residual (from the line of equality [$\text{ridge } \beta_{\text{hemodynamic}} = \text{ridge } \beta_{\text{VO}_2}$] with a slope of +1 or -1, depending on the clinical relation between a hemodynamic measure and peak VO_2) and used this residual distribution (standardized to variance 1) to calculate a *P* value for each individual protein's residual (via *Z* distribution). We assessed distribution of residuals used to evaluate extreme values using residual histograms and quantile-quantile plots. A similar approach was used to compare proteomic relations in 2 specific sets of phenotypes central to HFpEF: (1) PCWP/CO slope versus peak PVR; (2) peak exercise CO versus peak peripheral O_2 extraction. For this approach, we used a nominal $P < 0.05$, given this was a discovery effort.

Finally, to examine their clinical relevance, we tested the relation of these proteomic signatures of exercise response patterns to incident HF in FHS. Coefficients from ridge regression models developed on the basis of hemodynamic measures (above) in the CPET sample were multiplied by the individual protein levels in FHS participants to generate proteomic scores for each individual (that reflect the phenotype for which the ridge

model was built). These scores were applied in FHS in regression. Age and sex coefficients (used as independent variables in ridge regression) were excluded when we calculated the proteomic scores in FHS, ensuring that the resulting FHS proteomic scores were based on age/sex-adjusted protein-phenotype effect sizes. We used logistic regression to evaluate the association of each score with incident HF with adjustment for age and sex, initially, and then additional adjustment for clinical HF risk factors and BNP level. Multinomial logistic regression was used to evaluate the association of each score with HF subtypes (HFpEF/HFrEF/HF with unknown EF). We tested the association of 5 different CPET scores with incident HF, and we therefore specified a significance threshold of 0.05/5 scores (0.01). All analyses were performed in R (www.r-project.org).

RESULTS

CPET Sample

The CPET sample had a mean age of 62±11 years, was majority women (56%), with a mean body mass index of 30.5±7.0 kg/m², and a mean left ventricular EF of 65±7%. Individuals with HFpEF had a higher prevalence of smoking, atrial fibrillation, and use of beta blockers and exhibited poorer exercise performance (Table 1). Notably, only about half of the HFpEF group was taking diuretic medications, and although the mean resting PCWP was higher in the HFpEF group, the majority of individuals with HFpEF had resting PCWP below the clinical threshold of 15 mmHg. Finally, the arteriovenous oxygen difference was abnormally low on average (<14 mL/dL) in both the HFpEF and control groups, consistent with deficits in peripheral O₂ extraction in both groups.⁶

Integrative Analysis of the Cardiovascular Proteome and Exercise Responses

Peak VO₂ is an integrative measure of global cardiorespiratory fitness and was correlated with higher peak CO (Spearman $\rho=0.63$), lower PCWP/CO slope ($\rho=-0.58$), and peak PVR ($\rho=-0.49$; Figure 2). Peripheral O₂ extraction (related to peak CO and peak VO₂ by the Fick equation) was modestly associated with physiological exercise measures in this sample (with peak VO₂ $\rho=0.24$). Using ridge regression, we defined age- and sex-adjusted proteomic signatures for each of the 5 HFpEF exercise phenotypes (full coefficients in Table S2). Among individuals, proteomic scores derived from these signatures captured a significant proportion of the variance in the parent hemodynamic CPET phenotypes (ρ range, 0.59–0.77; Figure 2, Table S3). Finally, we observed a similar range of correlation of natriuretic peptides and supine PCWP with exercise responses, and, overall, the proteomic scores demonstrated higher correlation.

To evaluate proteins with the highest importance to each proteomic signature, we next selected the 50 proteins with the largest absolute magnitude ridge coefficients across each phenotype, observing significant heterogeneity in overlap of proteins across phenotypes (Figure 3A). Peripheral O₂ extraction displayed the most distinct proteomic signature, with 24 of its top 50 proteins being unique to that phenotype. To understand how specific proteins were related to distinct and overlapping phenotypes, we plotted the ridge coefficients and predicted secretion status and correlations among the proteins with the 50 highest-magnitude ridge coefficients that were shared across multiple phenotypes and those that were unique to peripheral O₂ extraction, PCWP/CO slope, and peak VO₂ (Figure 3B). The majority of proteins among the top 50 for each phenotype were shared across several phenotypes, including proteins implicated in regulation of key processes relevant to HFpEF such as inflammation (eg, numerous interleukins and tumor necrosis factor members) and fibrosis (eg, bone morphogenetic protein 6, thrombospondin-2, matrix metalloproteinases, fibroblast growth factor 23¹⁷).

Physiological Phenotyping and Cardiovascular Proteomics Identify Common and Distinct Pathways of Exercise Responses

In exploratory analyses, we prioritized molecular pathways relevant to HFpEF-defining phenotypes by examining each protein's ridge coefficient for a given phenotype ("importance" of that protein to the phenotype) in relation to its coefficient for peak VO₂—an integrated measure of fitness (Figure 4, Table S4). This construction enabled visualization of significant heterogeneity across the "peak VO₂ proteome" and proteomic correlates of each CPET phenotype. In general, most proteins related to central hemodynamic phenotypes (peak exercise CO, PCWP/CO slope, peak exercise PVR) had a similar magnitude of association with peak VO₂ (Pearson r magnitude range, 0.55–0.62). On the other hand, proteins related to peripheral O₂ extraction were generally not correlated with peak VO₂ ($r=0.03$), potentially owing to inclusion of control patients with symptoms of exertional intolerance in this study. The diversity of proteins important to peripheral extraction included proteins not necessarily related to cardiac processes (eg, kidney injury molecule-1: released by the kidney in response to injury,¹⁸ and hepatocyte growth factor: secreted by hepatocytes and exhibiting anti-inflammatory/antifibrotic properties¹⁹). In addition, proteins implicated in obesity regulation and cardiometabolic disease (eg, leptin, fatty acid-binding protein 4, insulin-like growth factor-binding protein-1/2) exhibited higher importance for peak VO₂ than for

Table 1. Characteristics of the Derivation Sample

Characteristic	Overall (N=151)	HFpEF (N=103)	Controls (N=48)
Age, y	62±11	63±11	61±10
Women	84 (56)	53 (52)	31 (65)
White race	145 (96)	97 (94)	48 (100)
Hypertension	105 (70)	73 (71)	32 (67)
Diabetes	22 (15)	18 (18)	4 (8)
Nonsmoker*	72 (48)	40 (39)	32 (68)
Prevalent coronary artery disease	33 (22)	26 (25)	7 (15)
Atrial fibrillation	28 (19)	24 (23)	4 (8)
ACE inhibitor use	39 (26)	26 (25)	13 (27)
Angiotensin receptor blocker use	22 (15)	15 (15)	7 (15)
Beta blocker use	68 (45)	53 (52)	15 (31)
Diuretic use	66 (44)	50 (49)	16 (33)
Body mass index, kg/m ²	30.5±7.0	30.8±7.4	29.9±6.1
Systolic blood pressure, mmHg	150±23	151±23	148±22
Diastolic blood pressure, mmHg*	78±12	78±12	78±11
Creatinine, mg/dL*	1.1±1.0	1.1±1.2	0.9±0.3
Left ventricular ejection fraction, %	65±7	65±8	65±6
PCWP, rest, mmHg	13.5±6.2	14.6±6.7	11.0±3.8
Mean PA pressure, rest, mmHg	17.9±5.4	19.1±5.9	15.2±2.7
Cardiac output, rest, L/min	5.2±1.6	5.0±1.7	5.7±1.4
Cardiac output, peak, L/min	11.8±3.9	10.6±3.3	14.3±4.0
PVR, rest, Wood units	1.0±1.5	1.1±1.7	0.8±0.9
PVR, peak, Wood units	1.6±1.2	1.8±1.4	1.1±0.5
C(a-v)O ₂ difference, rest, mL/dL	5.9±1.3	6.1±1.4	5.5±0.9
C(a-v)O ₂ difference, peak, mL/dL	11.6±2.0	11.6±2.1	11.5±1.9
Peak VO ₂ , mL/kg per min	15.9±5.1	14.1±3.8	19.7±5.4
PCWP/CO slope, mmHg/L per min	3.3±2.9	4.3±3.1	1.3±0.5
Peak work, watts	96.4±49.6	82.8±37.8	125.4±59.0
Peak respiratory exchange ratio	1.16±0.13	1.16±0.14	1.16±0.09

Values represent mean±SD for continuous variables and n (%) for categorical variables. ACE indicates angiotensin-converting enzyme; C(a-v)O₂ difference, arteriovenous oxygen difference; CO, cardiac output; HFpEF, heart failure with preserved ejection fraction; PA, pulmonary artery; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; and VO₂, oxygen uptake.

*Total N=149 for smoking, N=145 for creatinine, and N=150 for diastolic blood pressure.

peak exercise CO or PCWP/CO slope (direct measures of cardiac exercise performance). Indeed, apart from peak VO₂, leptin had the highest importance in peripheral O₂ extraction regressions, consistent with a role in modulating peripheral factors through obesity rather than central hemodynamic factors.

We next examined our results for 2 major physiological etiologies of elevated pulmonary artery pressures in HFpEF: (1) postcapillary elevation in left sided filling pressures (PCWP/CO slope), and (2) pre-capillary pulmonary vascular dysfunction (peak PVR; Figure 5A).^{20–46} Strikingly, higher levels of growth differentiation factor 2/bone morphogenetic protein 9 and insulin-like growth factor-binding protein-3 were more strongly related (higher positive ridge coefficient) to peak exercise PVR versus PCWP/CO slope, consistent with demonstrated clinical, genetic, and

mechanistic roles for these proteins in pulmonary arterial hypertension.^{20,21} We also identified several proteins not widely implicated in pulmonary hypertension previously but with high importance for peak PVR (relative to PCWP/CO slope), including mediators implicated in inflammation (chitotriosidase-1,²² monocyte chemoattractant protein-4²⁵) and fibrosis (eg, osteoclast-associated immunoglobulin-like receptor,²³ plasminogen activator inhibitor-1²⁴). On the other hand, proteins with a demonstrated role in myocardial fibrosis and HF risk (eg, metalloproteinase inhibitor 4,³⁹ fatty acid-binding protein 4,⁴² growth differentiation factor-15³⁷) were more strongly related to PCWP/CO slope than peak exercise PVR, as were proteins putatively linked to myocardial metabolism and lipid accumulation (eg, receptor for advanced glycation end products [RAGE] and its ligand EN-RAGE,³⁸ lipoprotein lipase and its

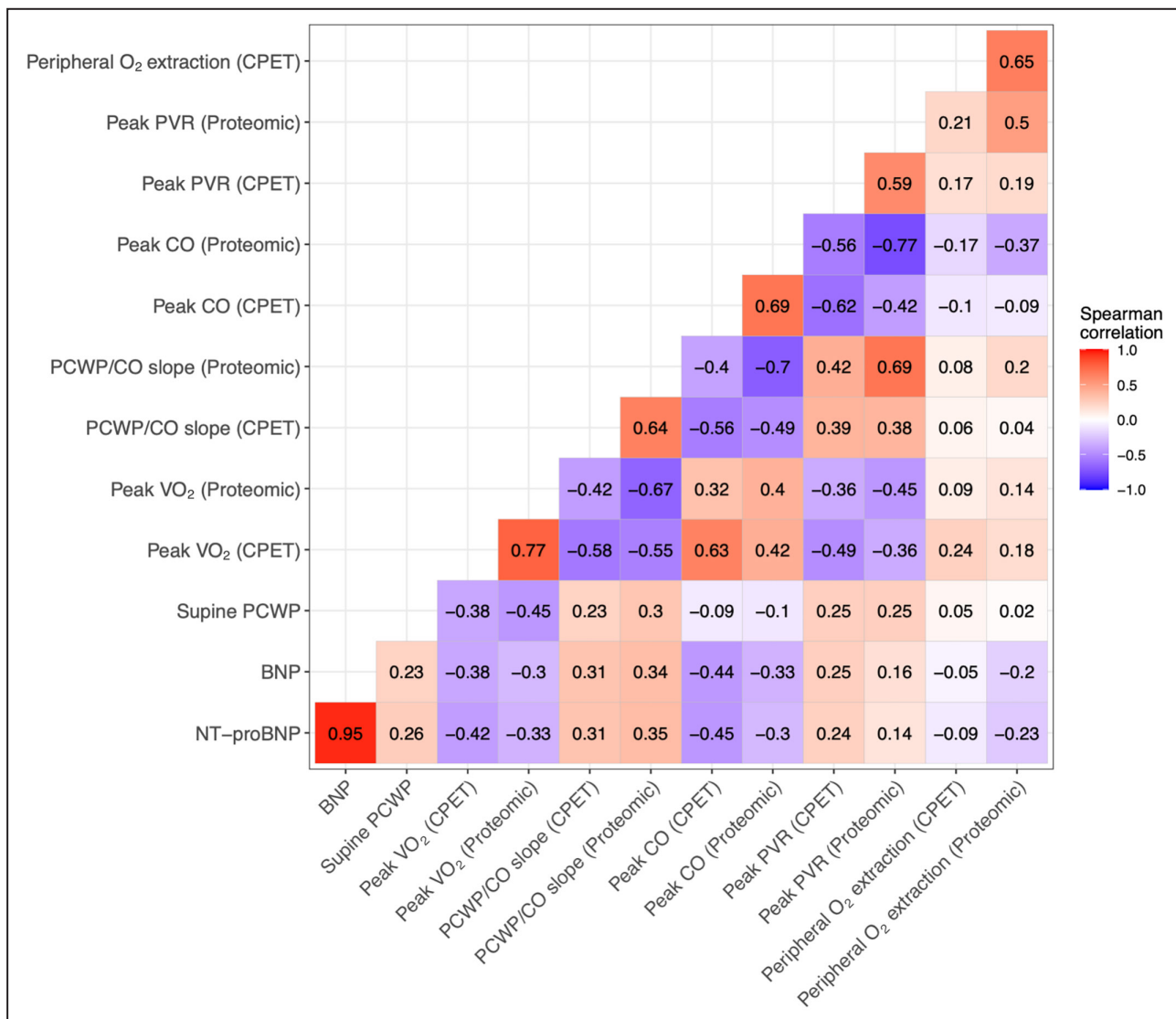


Figure 2. Spearman correlations of parent physiological/hemodynamic exercise measures, proteomic scores, natriuretic peptides, and resting supine PCWP.

Exercise measures are labeled “CPET,” proteomic scores are labeled “proteomic.” BNP and NT-proBNP were measured by Olink. Correlation *P* values are provided in Table S3. BNP indicates B-type natriuretic peptide; CO, cardiac output; CPET, cardiopulmonary exercise test; NT-proBNP, N-terminal pro-B-type natriuretic peptide; O₂, oxygen; PCWP, pulmonary capillary wedge pressure; PCWP/CO, pulmonary capillary wedge pressure to cardiac output ratio; PVR, pulmonary vascular resistance; and VO₂, oxygen uptake.

inhibitor angiotensin-like protein³⁴⁴) and pulmonary congestion (eg, pulmonary surfactant-associated protein D [PSP-D]: alveolar protein released in the presence of lung inflammation or injury⁴⁶).

We finally turned to 2 measures that directly determine peak VO₂: peak exercise CO and extraction (Figure 5B).[‡] Proteins that displayed higher importance for peripheral O₂ extraction versus peak CO reflect mechanistic processes not confined to the heart: inflammation and oxidative stress (paraoxonase³⁴¹), vascular health and hemostasis (tissue plasminogen activator⁵¹), metabolic dysfunction (adenosine deaminase,³¹ low-affinity

immunoglobulin gamma Fc region receptor-II,⁵⁰ interleukin-27⁵³); fibrosis (matrix metalloprotein-7/10,⁴⁷ urokinase plasminogen activator⁵²), and skeletal muscle health and iron metabolism (transferrin receptor protein 1⁴⁸).

Proteomic Signatures of Exercise Response Phenotypes Are Associated With Higher Risk of HFpEF

In the FHS (N=522; median follow-up, 12.4 [interquartile range, 7.1–16.5]) years, age 69±8 years, 45% women (additional characteristics in Table S5), there were 122 individuals who developed HFpEF, 94 with HFREF, and 45 had unknown EF. We evaluated the associations of the

[‡]References: 17,20,25,31,32,35,41,47–61.

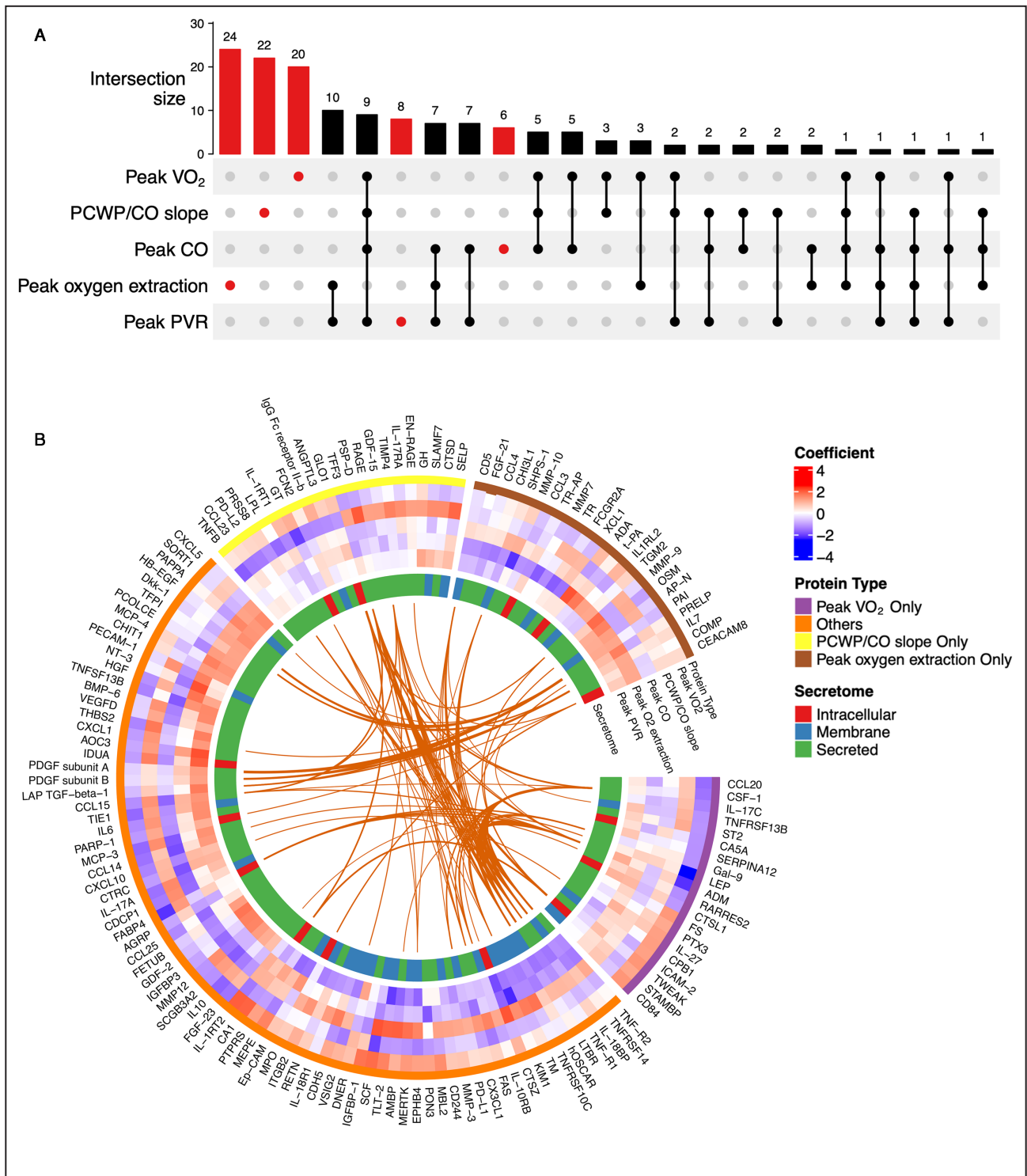


Figure 3. Shared and distinct associations of proteins and exercise phenotypes.

A, Shared and distinct associations of the top 50 proteins (by absolute magnitude of ridge coefficient) for each phenotype. **B**, Circos plot organizing these top 50 proteins across exercise response phenotypes. The outermost color bar signifies the phenotype; the heatmap expresses the ridge model coefficients (scaled); and the inner color bar represents the secreted status of the protein. The brown line connections and their width signify absolute magnitude Spearman correlation >0.6 between proteins of interest with different phenotype associations. Secretome protein annotation was performed using data from the Human Protein Atlas project. CO indicates cardiac output; PCWP/CO, pulmonary capillary wedge pressure to cardiac output ratio; PVR, pulmonary vascular resistance; and VO₂, oxygen uptake. Expanded protein names are available in [Table S1](#).

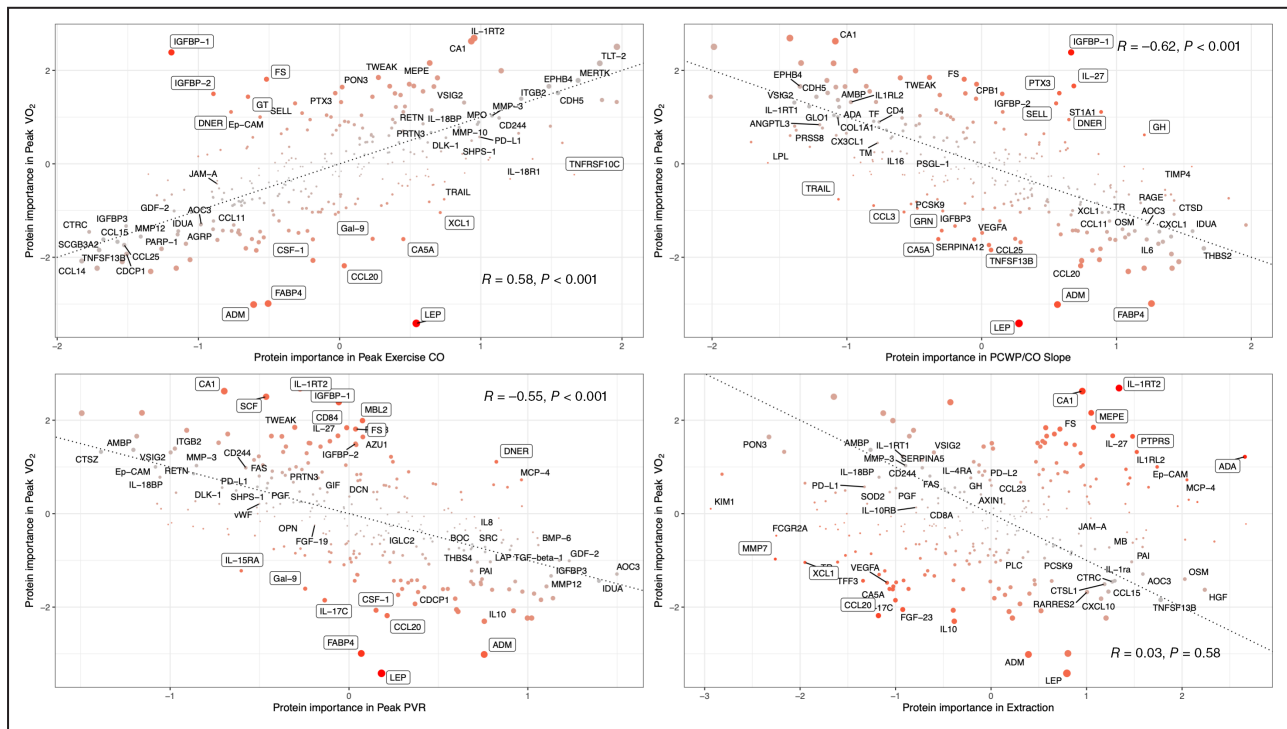


Figure 4. Shared and distinct protein importance (ridge model coefficient) across phenotypes.

The Pearson correlation coefficient is displayed. The ridge coefficients are multiplied by 100 for plotting. Our intention was to label proteins close to the line of equality (dotted line, “shared” mechanisms) and those that had a significant residual from this line (nominal $P < 0.10$). Those proteins that are encircled had a more extreme residual ($P \leq 0.05$), signifying “distinct” importance for 1 phenotype over another. The size of each point corresponds to its magnitude for importance for peak VO_2 (scaled), and its color represents magnitude of residual (with red as higher residual). Some points may not be labeled due to overplotting. CO indicates cardiac output; PCWP/CO, pulmonary capillary wedge pressure to cardiac output ratio; PVR, pulmonary vascular resistance; and VO_2 , oxygen uptake. Expanded protein names are available in [Table S1](#).

proteomic signatures of specific exercise phenotypes with incident HF subtypes in FHS ([Figure 6](#), [Table S6](#)). In models adjusted for clinical risk factors and B-type natriuretic peptide levels, a 1-SD higher peak VO_2 proteomic score was associated with a 33% lower odds of developing overall HF or HFpEF (P less than pre-specified Bonferroni-adjusted threshold of 0.01 for both) and a 30% lower odds of developing HFrEF ($P = 0.025$, which did not meet statistical significance). A higher PCWP/CO proteomic score was associated with a 43% to 44% higher odds of developing HF, HFpEF, and HFrEF for each 1-SD increase in the score ($P \leq 0.010$ for all).

DISCUSSION

Prior efforts focused on mechanistic discovery in HFpEF have relied on a clinical diagnosis of HFpEF (with inherent limitations due to pathophysiological heterogeneity) or animal systems that do not necessarily recapitulate human biology.³ In this study, we united cardiovascular proteomics with exercise responses across carefully phenotyped individuals with and without HFpEF with the dual priorities of identifying shared and distinct pathways of HFpEF pathogenesis and

developing resting molecular signatures of these exercise responses capable of stratifying risk of incident HFpEF. We identified a broad cardiovascular proteomic architecture largely shared between peak VO_2 and phenotypes reflecting “central” cardiovascular reserve (PCWP/CO slope, peak exercise CO, peak exercise PVR), but not peripheral O_2 extraction, which is also recognized to be impaired in HFpEF.⁶ As a testament to the validity of this phenotypic-proteomic approach for identifying underlying physiological heterogeneity, we identified pathways with a greater importance to peak PVR in concert with extensive prior mechanistic work and therapeutic implication (eg, growth differentiation factor 2), as well metabolic pathways implicated in central cardiac function (eg, growth differentiation factor-15, fatty acid-binding protein 4) more important to left heart performance. These findings were accompanied by a host of novel pathways shared and distinct across exercise response phenotypes with mechanistic plausibility but heretofore not widely described in HFpEF. Of note, the proteomic architecture of peripheral extraction was more distinct from that of peak VO_2 , including a set of proteins important in vascular health/hemostasis, general metabolic health, noncardiac organ injury,

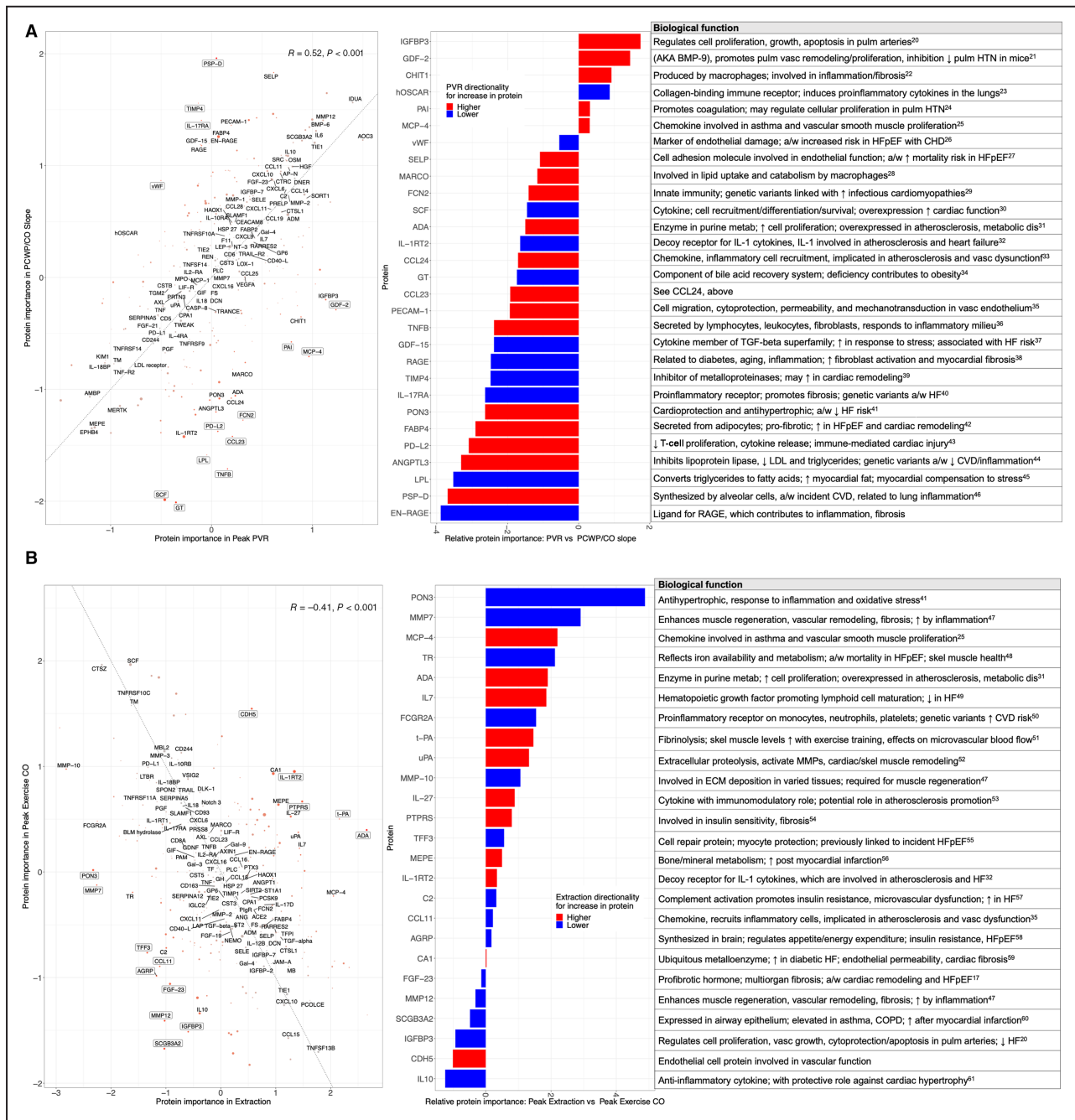


Figure 5. Selected phenotypes of central importance in HFpEF physiology.

A (left) plots the protein importance (ridge model coefficient) of PCWP/CO slope (left heart filling with exercise) against peak exercise PVR (pulmonary hypertension). Labeling scheme as in Figure 4, with caution that all points may not be labeled to avoid overplotting. The middle panel displays a subset of proteins with significant residual ($P < 0.10$). Each bar is calculated as a log of the ratio of the magnitude of a protein's importance to PVR divided by its importance to PCWP/CO slope. Therefore, a ratio > 1 here implies a higher magnitude ridge coefficient for PVR relative to ridge coefficient for PCWP/CO slope (and the log of that ratio > 0 , or to the right of 0 on the x axis). Effectively, those proteins to the right of 0 are more highly weighted on peak exercise PVR, while proteins to the left of 0 are more highly weighted on PCWP/CO slope. The color represents the sign of the ridge coefficient for PVR (such that an increase in protein would be related to a higher peak exercise PVR if red). The left panel reflects biological annotations. PCWP/CO, pulmonary capillary wedge pressure to cardiac output ratio; PVR, pulmonary vascular resistance; pulm, pulmonary; AKA, also known as; a/w, associated with; CHD, coronary heart disease; CVD, cardiovascular disease; dis, disease; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HTN, hypertension; LDL, low-density lipoprotein cholesterol; vasc, vascular.

B represents a similar plot for peak exercise CO and peak peripheral O_2 extraction. The color bars represent the ratio of the importance in extraction to importance to peak exercise CO. a/w indicates associated with; CO, cardiac output; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; ECM, extracellular matrix; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; MMPs, matrix metalloproteinases; skel, skeletal; and vasc, vascular. Expanded protein names are available in Table S1.

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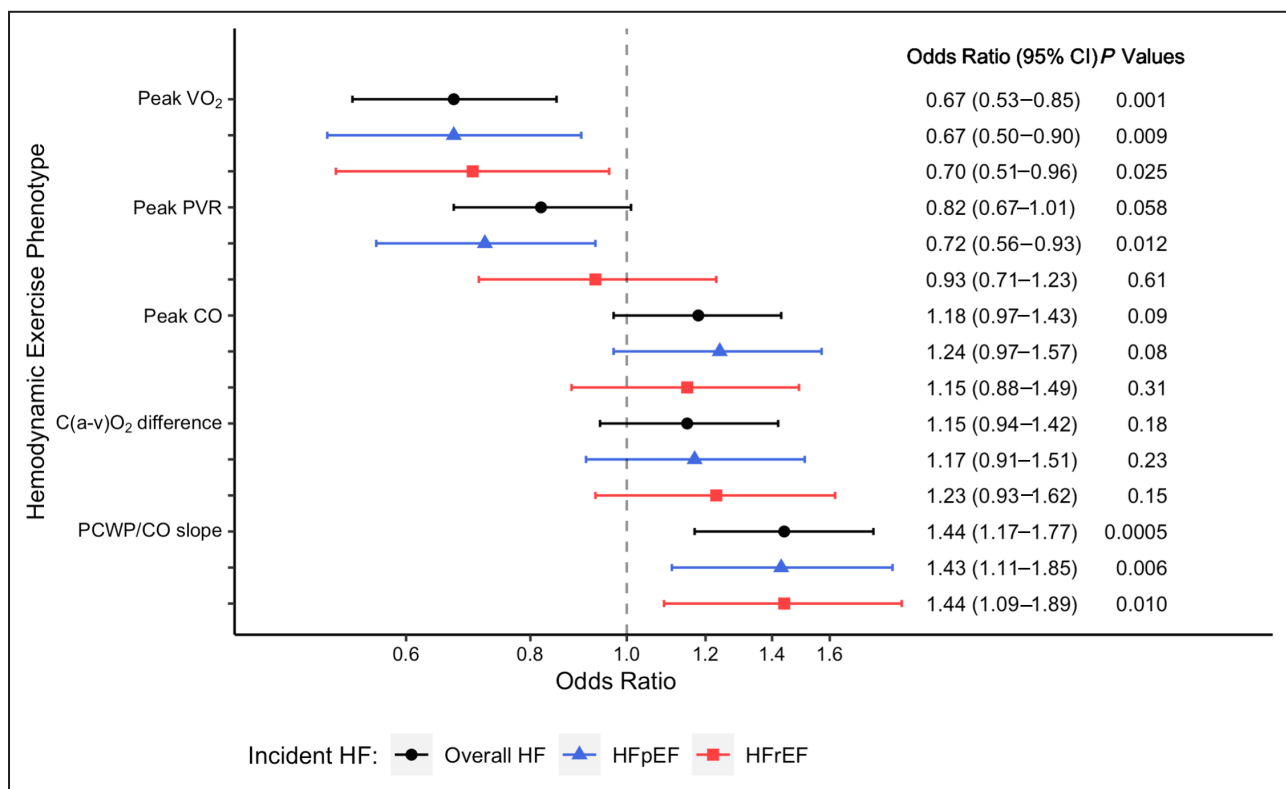


Figure 6. Prospective associations of proteomic signatures of exercise phenotypes with incident HF subtypes in Framingham Heart Study.

The association of each proteomic signature with overall HF and with HFpEF and HFrEF are displayed. Logistic regression models were used to relate proteomic scores to overall HF, and multinomial logistic regression was used to relate the proteomic scores to HFpEF and HFrEF. Models were adjusted for age, sex, smoking, diabetes, prevalent coronary heart disease, serum creatinine, body mass index, systolic blood pressure, use of antihypertensive medications, and B-type natriuretic peptide level. C(a-v)O₂ difference indicates arteriovenous oxygen difference; CO, cardiac output; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; PCWP/CO, pulmonary capillary wedge pressure to cardiac output ratio; PVR, pulmonary vascular resistance; and VO₂, oxygen uptake.

and iron metabolism, consistent with a less “central cardiac” cause of this limitation in HFpEF. Finally, our proteomic signatures for peak VO₂ and PCWP/CO slope were associated with incident HFpEF in the FHS, independent of known predictors of HF. These results represent a first demonstration of the utility of precision proteomic and exercise hemodynamic phenotyping to define and prioritize distinct functional biomarkers and pathways in HFpEF, a key step in focusing future efforts in basic and clinical studies of this growing, heterogeneous condition.

This work represents a unique approach in its integration of precise phenotypes (including invasive hemodynamics), exercise, and the circulating cardiovascular proteome to prioritize pathways with potential relevance to HFpEF. We used this paradigm to address potential underlying mechanisms of 2 distinct physiologies that often coexist in HFpEF: pulmonary vascular dysfunction and exaggerated increase in left heart filling pressures.⁷ While growth differentiation factor 2 and insulin-like growth factor-binding protein-3 (both

weighted more heavily on exercise PVR than PCWP/CO slope) have previously been implicated in pulmonary arterial hypertension,^{20,21} we also identified inflammatory (eg, chitotriosidase-1,²² monocyte chemoattractant protein-4²⁵) and profibrotic (eg, osteoclast-associated immunoglobulin-like receptor, plasminogen activator inhibitor-1²⁴) proteins to have greater potential relevance to peak exercise PVR (relative to left heart filling during exercise). By contrast, pathways central to myocardial fibrosis (eg, fatty acid-binding protein 4 and growth differentiation factor-15) and myocardial metabolism (eg, RAGE, EN-RAGE, lipoprotein lipase, angiotensin-like protein 3) appeared more highly weighted on left heart performance (PCWP/CO slope) over PVR, consistent with the contribution of myocardial stiffness and abnormal energetics during HFpEF.³ In addition, several proteins not previously described in left heart filling limitation during HF more broadly (eg, PSP-D, an alveolar protein released in the setting of lung injury⁴⁶) appeared more closely related to PCWP/CO than peak PVR, suggesting avenues for

investigation of subclinical pulmonary edema and exertional limitation in the context of elevated postcapillary pulmonary pressures.

A second key observation from our work was that the proteomic correlates of peripheral O_2 extraction appeared to be largely distinct from those of more “central cardiac” exercise phenotypes. While impaired peripheral O_2 extraction is an established contributor to exertional intolerance in HFpEF,⁶ its proteomic signature is unique and features proteins with noncardiac origin (eg, kidney injury molecule 1, hepatocyte growth factor), established links with metabolic stress (leptin, fatty acid-binding protein 4, insulin-like growth factor-binding protein-1 and -2), involvement in iron metabolism (eg, TR), and organ fibrosis (eg, urokinase plasminogen activator, matrix extracellular phosphoglycoprotein, matrix metalloprotein-7 and -10). These findings are consistent with the noncardiac, heterogeneous physiology of impaired peripheral O_2 extraction and its relation to metabolic risk (eg, obesity, hypertension, diabetes in HFpEF⁶).

Beyond proteins with disparate relations across phenotypes, proteins with similar importance for both peak VO_2 and cardiocentric responses included those with well-known functions in inflammation and matrix remodeling (interleukin-1RL1 [ST2], interleukin-6, matrix metalloprotein-12, chemokine [C-C motif] ligand 15, chemokine [C-C motif] ligand 7/monocyte chemoattractant protein 3, thrombospondin-2). As with other analyses, this approach also highlighted several pathways not previously reported in HFpEF but with biological plausibility, including lysosomal metabolism (iduronidase), leukocyte trafficking, extravasation, and immune activation (amine oxidase copper containing-3/vascular adhesion protein 1, TREM-like transcript 2), myocardial-vascular remodeling (tyrosine-protein kinase receptor Tie-1, ephrin type-B receptor 4), mitophagy (proto-oncogene tyrosine-protein kinase MER⁶²), angiogenesis and cell differentiation (stem cell factor³⁰), and pulmonary processes (PSP-D), among others. Indeed, these results are consistent with alteration in myocardial expression of several genes encoding these proteins (eg, decreased expression of proto-oncogene tyrosine-protein kinase MER, ephrin type-B receptor 4; increased expression of galectin-9) in human HFpEF in a recent seminal report.¹¹ Importantly, the majority of proteins identified as being relevant to cardiocentric exercise responses in our study are predicted to be secreted, and future studies (including multisite sampling) are warranted to interrogate specific cell types and tissues of release and action.

The current study also addressed the important need for better clinical biomarkers of HFpEF, especially earlier in its course. Not only have prior studies of HFpEF-specific biomarkers been met with mixed results,^{13,63–65} but established biomarkers generally

have a stronger association with incident HFrEF versus HFpEF. Given the pathophysiological heterogeneity underlying HFpEF that may partially explain this finding,⁶³ recent proteomic discovery efforts targeted specific HFpEF comorbidity¹³ or individuals with a clinical diagnosis of HFpEF.^{64,65} While it is difficult to directly compare our results with other proteomic efforts in HFpEF due to differences in the analytic approach and proteomic coverage, our findings identified many pathways (eg, inflammation, fibrosis, kidney injury, cardiac stress) previously implicated in studies of individuals with overt HFpEF.^{13,64,65} Our findings add to this prior literature by providing insight into how these pathways relate to specific physiologies as assessed by exercise testing. As such, we leveraged key clinically relevant exercise responses in HFpEF^{4,8} to generate a potentially generalizable, functional biomarker of HFpEF risk. In 500 FHS participants followed for \approx 12 years, the proteomic signatures of greater peak VO_2 (global cardiorespiratory fitness) and lower PCWP/CO slope (reflecting left ventricular–left atrial response to exercise) were associated with lower risk for overall HF and HFpEF. While the statistical significance of the relations with HFrEF was attenuated compared with HFpEF, this may result from statistical power. It is notable that the effect estimates were largely consistent across HF subtypes. Intriguingly, these findings indicate that proteomic instruments of peak VO_2 and PCWP/CO slope may offer utility not only in describing mechanisms of disease but also in forecasting HFpEF propensity.

The findings from the present investigation are especially relevant given (1) the difficulty in obtaining invasive assessments of HFpEF that are defining for the disease, and (2) the emergence of metabolic therapies that may help to both prevent and treat HFpEF.^{66,67} Indeed, broader application of precision exercise phenotyping could have important clinical implications in HFpEF as distinct phenotypes may respond differently to therapy. For example, exercise training has been demonstrated to lead to improvements in peripheral O_2 extraction, but not in CO augmentation, in HFpEF.⁶⁸ Therefore, identifying subgroups of HFpEF patients with peripheral impairments may inform exercise recommendations. Additionally, blood-based biomarkers of exercise phenotypes linked to future HFpEF risk may help to diagnose HFpEF before overt congestion, thereby improving risk prediction and targeting potential therapies to specific physiological abnormalities identified through exercise.

While we recognize that these observations (like any published study solely conducted in human tissue/biofluid) are not definitive for any given mechanism, they uniquely use exercise-based functional responses characteristic of HFpEF to make observations that represent an important first step in prioritizing pathways for further mechanistic and clinical study. However, several

limitations merit comment. A key feature of most proteomic studies is the limitation of the queried proteomic space and potential for overfitting given larger proteome relative to sample size. Certainly, broader proteomic coverage and greater sample size may expand potential novel discovery, and the current project offers a paradigm to interpret these data in the future. While a natural consequence of this work may be use of broader proteomic markers for prediction of HF (potentially even of HFpEF), the current work is limited by proteomic space and sample size to address this in a scientifically rigorous fashion. In addition, the limitation of our proteomic space to those proteins that were highly expressed may limit our ability to detect low-abundance proteins that may have important physiological effects. Larger studies with broader proteomic coverage are currently under way to address these limitations. The limited proteomic coverage also challenged standard pathway analyses, rendering a literature-based curation necessary. This approach has been successful in prior large proteomics efforts⁶⁹ and avoids fundamental limitations in pathway specification in the setting of incomplete proteomic coverage. We also acknowledge that selection of the top 50 proteins (by variable importance) for each exercise phenotype was an arbitrary threshold intended to prioritize the most important proteins for comparison among phenotypes and therefore should be viewed as exploratory. It is important to note that proteins with “adverse” ridge coefficients for a given phenotype cannot distinguish whether expression of these proteins are “compensatory” (higher levels as a consequence of the hemodynamic perturbation) or “causative” (higher protein levels cause the abnormality), a phenomenon known as reverse causation. Nevertheless, these results are to be viewed as a unique way to prioritize important proteins for downstream study, not to determine their role in HFpEF.

Importantly, our derivation sample was a case-control study among stable outpatients with exertional dyspnea and hemodynamic evidence of elevated left heart pressures at the same time that blood sampling was obtained. This form of HFpEF may not be representative of more severe manifestations of decompensated HFpEF. In particular, only about half of individuals with HFpEF were prescribed standing diuretics, suggesting that our findings may not be generalizable to individuals with HFpEF and overt resting congestion and may differ from populations selected for clinical trials, which may be purposely enriched for individuals with resting congestion. By analyzing a variable that was also used to define HFpEF status in our primary analyses (PCWP/CO), our approach may be open to an element of “circularity.” However, we counteract this by relating individual proteins to PCWP/CO modeled continuously without accounting for HFpEF status and then measure the association of this proteomic score

with future HFpEF in a separate sample with HFpEF ascertained clinically. Moreover, our control participants underwent exercise testing for evaluation of dyspnea and therefore reflect individuals who are less healthy than the general population. Nevertheless, association of proteomic scores with future HFpEF in the FHS suggests clinical applicability, and reliance on hemodynamic confirmation of elevated left heart filling pressures with exercise was a uniform diagnostic criterion, often challenging in HFpEF due to variable disease presentation.⁸ Whether the observed proteomic signatures of exercise responses are unique to HFpEF or shared across HF subtypes (eg, HF with reduced EF) warrants further study. Finally, our study samples comprised mostly White individuals of European descent, and future studies are necessary to expand findings to more diverse populations.

In conclusion, we related a broad cardiovascular proteome to 5 precise exercise phenotypes that characterize HFpEF, identifying both shared and unique pathways relevant to HFpEF pathogenesis. Proteomic instruments based on invasively determined hemodynamic indices were associated with future HFpEF in an at-risk population. The current investigation underscores the importance of precision exercise phenotypes in guiding molecular discovery and risk assessment, including identification of potentially novel targets for mechanistic interrogation and therapeutic potential in HFpEF.

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Disclosures

Dr Shah has served as a consultant for Myokardia, Cytokinetics, and Best Doctors, and has been on a scientific advisory board for Amgen. Dr Shah is a coinventor on a patent for ex-RNAs signatures of cardiac remodeling. Dr Murthy has stock holdings in General Electric and receives research funding from Siemens. Dr Ho has received past research grant support from Bayer, AG. Dr Lewis acknowledges research funding from the National Institutes of Health and the American Heart Association as well as Amgen, Cytokinetics, Applied Therapeutics, AstraZeneca, Sonovie, NXT, and RIVUS in relation to projects and clinical trials investigating exercise capacity that are distinct from this work. He has served as a scientific advisor for Pfizer, Merck, Boehringer-Ingelheim, Novartis, American Regent, Relypsa, Cycleron, Cytokinetics, RIVUS, Applied Therapeutics, and Amgen and receives royalties from UpToDate for scientific content authorship related to exercise physiology. Dr Naylor has received speaking honoraria from Cytokinetics. The opinions expressed in this manuscript are those of the authors and do not necessarily represent those of the National Institutes of Health or the Department of Health and Human Services. The remaining authors have no disclosures to report.

Supplemental Material

Tables S1–S6

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