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# Proteomic Signatures of Heart Failure in Relation to Left Ventricular Ejection Fraction

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## ABSTRACT

**BACKGROUND** There is a growing recognition of the inherent limitations of the use of the left ventricular ejection fraction (LVEF) to accurately phenotype patients with heart failure (HF).

**OBJECTIVES** The authors sought to identify unique proteomic signatures for patients with HF with reduced ejection fraction (HFrEF), HF with a midrange LVEF (HFmrEF), and HF with preserved ejection fraction (HFpEF), as well as to identify molecular differences between patients with ischemic and nonischemic HF.

**METHODS** We used high-content aptamer-based proteomics technology (SOMAscan) to interrogate the blood proteome of age- and sex-matched patients with HF within different LVEF groups.

**RESULTS** Within the Washington University Heart Failure Registry, we identified age/sex-matched patients within 3 LVEF categories: HFrEF (LVEF <40%), HFmrEF (LVEF 40% to 50%), and HFpEF (LVEF >50%). We found that patients with HFrEF, HFmrEF, and HFpEF had unique variations in circulating proteins that reflected distinct biological pathophysiologies. Bioinformatics analysis revealed that there were biological themes that were unique to patients with HFrEF, HFpEF, or HFmrEF. Comparative analyses of patients with HFmrEF with improved LVEF and patients with HFmrEF with unchanged LVEF revealed marked differences between these 2 patient populations and indicated that patients with recovered LVEF are more similar to patients with HFpEF than to patients with HFrEF. Moreover, there were marked differences in the proteomic signatures of patients with ischemic and nonischemic HF.

**CONCLUSIONS** Viewed together, these findings suggest that it may be possible to use high-content multiplexed proteomics assays in combination with the clinical assessment of LVEF to more accurately identify clinical phenotypes of patients with HF. (J Am Coll Cardiol 2020;76:1982–94) Published by Elsevier on behalf of the American College of Cardiology Foundation.

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The use of left ventricular ejection fraction (LVEF) to stratify patients with heart failure (HF) is currently the most accurate clinical method to identify those patients with HF who are more likely to respond favorably to neurohormonal antagonists and medical devices (1). Nonetheless, there is growing awareness in the field of the need to phenotype patients with HF beyond the conventional assessment of LVEF (2). As one example, patients with HF with a history of a reduced LVEF ≤40% (HFrEF) who recover LV function (LVEF ≥50%) on evidence-based medical and device therapies would no longer qualify for the use of diuretics and neurohormonal antagonists based on the current guideline recommendations for treating patients with HFrEF (3). However, the result of the TRED-HF (A Pilot Feasibility Study in Recovered Heart Failure) trial demonstrated that discontinuation of evidence-based medical therapies in patients with HFrEF with a recovered LVEF ≥50% culminates in worsening HF (4). Thus, in the modern era of HF therapeutics, the assessment of LVEF in isolation does not necessarily provide accurate information with respect to the phenotype of a patient with HF.

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The development of high-throughput molecular biology techniques (omics) provides the opportunity to deep phenotype patients with HF and to increase our understanding of the pathophysiology of HF (reviewed in [5]). In the current study, we have used an aptamer-based proteomics technology, SOMAscan, to interrogate the blood proteome of age- and sex-matched patients with HF who were classified based on their LVEF into HFrEF (LVEF <40%), HF with a midrange LVEF (HFmrEF [LVEF 40% to 50%]), and HF with preserved LVEF (HFpEF [LVEF >50%]). Here we demonstrate that is feasible to use a high-content multiplexed assay to identify distinct proteomic signatures for patients with HFrEF, HFmrEF, and HFpEF, as well as to identify molecular differences between ischemic and nonischemic patients with HF.

## MATERIALS AND METHODS

**SUBJECTS AND SAMPLES.** The patients for these studies represent a subset of patients with HFrEF (LVEF <40% [n = 47]), HFpEF (LVEF >50% [n = 43]), and HFmrEF (LVEF 40% to 50% [n = 83]), who were selected randomly from a larger cohort of previously reported (6) age- (± 5 years) and sex-matched patients with HFrEF (LVEF <40% [n = 102]), HFpEF (LVEF >50% [n = 82]), and HFmrEF (LVEF 40% to 50% [n = 168]) enrolled in the Washington University Heart Failure Registry, using a computer-generated random selection algorithm. The Washington University Heart Failure Registry is a prospective registry of inpatients and outpatients with clinical evidence of HF, irrespective of LVEF (7). Detailed patient information along with a blood sample were prospectively collected at the time of enrollment (March 2010 to August 2013) into the registry, and patient vital status was followed for 2 years after enrollment (7). The registry and the analysis included in this study were approved by the Washington University Institutional Review Board entitled "Washington University Heart Failure Registry." As described previously, the patients with HFmrEF were further categorized into HFmrEF-improved (n = 59), HFmrEF unchanged (n = 8), or HFmrEF deteriorated (n = 16), based on whether the LVEF at the time of enrollment into the registry was improved, worsened, or the same as a prior assessment of LVEF that was obtained at the time the patient was first diagnosed with HF, which was determined from a retrospective chart review. Because there were too few patients in the HFmrEF deteriorated and HFmrEF unchanged groups, for the purpose of subgroup analysis, we combined these patients into HFmrEF-unimproved group (n = 24). The cohorts of patients with HFrEF and patients with HFpEF in this study were propensity matched for sex and age with the HFmrEF cohort, as described (6).

**PLASMA PROTEOMIC PROFILING, DATA PREPROCESSING, AND BIOINFORMATICS ANALYSES.** See the Supplemental Appendix for details.

**STATISTICAL ANALYSIS.** See the Supplemental Appendix for details.

## RESULTS

**INTERNAL AND BIOLOGICAL VALIDATION OF THE SOMAscan PROTEIN EXPRESSION DATA.** The results of the SOMAscan protein expression data were internally validated for precision and accuracy. Precision was assessed based on replicate analysis and accuracy was assessed based on expected changes in plasma proteins across specific patient subgroups. We ran 5 replicates of a vendor-provided calibrator sample and 3 replicates of pooled plasma on each of the 8 assay plates we analyzed. The coefficient of variation (CV) for the vendor-provided calibrators was <3.5% among 50% of SOMAmer reagents, and <12.4% among 95% of SOMAmer reagents. The median CV from pooled plasma samples was 5.2% across the 8 plates of the SOMAscan assays. This analysis confirmed the

### ABBREVIATIONS AND ACRONYMS

**ELISA** = enzyme-linked immunosorbent assay

**HF** = heart failure

**HFmrEF** = heart failure with a midrange ejection fraction

**HFpEF** = heart failure with a preserved ejection fraction

**HFrEF** = heart failure with a reduced ejection fraction

**LVEF** = left ventricular ejection fraction

**PCA** = Principal Components Analysis

reproducibility (precision) of the measurements reported herein.

To assess the accuracy and biological relevance of protein measurements, we analyzed the expression of coagulation factors in patients treated with warfarin, a compound that inhibits the synthesis of vitamin K-dependent clotting factors, as well as the relationship between the putative kidney function marker CST3 and plasma creatinine. Supplemental Figures 1A and 1B illustrate that patients treated with warfarin, as expected, had significantly lower levels of the vitamin K-dependent coagulation factors F2, F7, F9, and F10, protein C, and protein S. In addition, Supplemental Figures 1C and 1D demonstrate that the plasma levels of the putative kidney function marker CST3 correlated well with plasma creatinine levels.

The verified positive response to warfarin elucidated the need for a source of variation analysis to identify potential confounders for the HF cohort analysis. As anticipated, age and sex, which were matched across the cohorts, contributed relatively little to the overall variation (Supplemental Figure 2). Similarly, the use of warfarin, calcium-channel blockers, and other related medications (angiotensin-converting enzyme inhibitors, beta-blockers, dyslipidemia drugs) contributed little to the signal between cohorts. Further, LVEF as a continuous variable contributed approximately 1% to the overall variability of the HF proteome. In contrast, HF etiology (ischemic vs. nonischemic) contributed between 5.8% and 14.1% of the observed variance. The largest component of the variance in the analysis was unassigned, indicating an as-of-yet undetermined source.

To further validate the accuracy of the SOMAscan analysis, we performed enzyme-linked immunosorbent assays (ELISAs) on 3 proteins whose expression levels varied among the patients with HFrEF, HFmrEF, or HFpEF, namely cystatin C, soluble ST2 (sST2), and C-reactive protein. As shown in Supplemental Figure 1E, the expression levels of the analytes determined by the SOMAscan and the protein values determined by ELISA were closely correlated across the range of proteins that were tested: cystatin C, $r = 0.94$ ($p < 0.001$); ST2, $r = 0.95$ ($p < 0.001$), and C-reactive protein, $r = 0.95$ ($p < 0.001$).

**PATIENT CHARACTERISTICS.** Table 1 displays the baseline characteristics of the 173 subjects included in the study. As shown, the age for the entire cohort of patients with HF was 55.3 ± 12.6 years, with 58% male patients, 73% Caucasian, and 24% African American. The major comorbidities included hyperlipidemia (43%), hypertension (55%), diabetes (25%), coronary artery disease (24%), obstructive sleep apnea (27%), and chronic obstructive pulmonary disease (14%). The distribution of New York Heart Association functional class for the entire cohort was 14% class I, 55% class II, 23% class III, and 8% class IV; 86% of patients were on a beta-blocker and 85% of the patients were on an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker. The baseline demographics for the patients with HFrEF, HFmrEF, and HFpEF were relatively well matched, with the exception that there were more patients with ischemic heart disease in the HFrEF subgroup. The etiologies for the HF in the different patient cohorts are shown in Table 2.

**PROTEOMIC PROFILING OF HEART FAILURE PATIENTS STRATIFIED BY LV EJECTION FRACTION.** We performed a 3-dimensional principal component analysis (PCA) of the proteomic profiles for the patients with HFrEF, HFmrEF, and HFpEF. As shown in Figure 1, the patients with HFpEF, HFrEF, and HFmrEF partitioned into 3 partially overlapping clusters (8). The centroid location was then computed for each of the 3 cohort groups within the first 3 component spaces, and the intercohort Euclidean distance of separation between the centroids of the patients with HFrEF, HFmrEF, and HFpEF was determined. As shown in Figure 1A, the distance between the cluster centroids was 4.2 U for HFmrEF versus HFpEF, 5.2 U for HFrEF versus HFpEF, and 6.2 U for HFmrEF versus HFrEF. The unique distances between the centroids for the different clusters suggests that the plasma proteomic profiles were most similar for patients with HFmrEF and HFpEF, and were most dissimilar for patients with HFmrEF and HFrEF. These latter findings were intriguing, insofar as approximately 70% of the patients in the HFmrEF group were patients with HFrEF whose LVEF increased on medical and/or device therapies. To further explore these findings, we stratified the HFmrEF patients into HFmrEF-improved and HFmrEF-unimproved subgroups (see Supplemental Table 1 for demographics of these subgroups). Figure 1B shows that the centroid for the cluster of the HFmrEF-unimproved patients was approximately equidistant between the centroids for the HFrEF cluster at 3.3 U and the HFpEF cluster at 3.2 U, whereas the centroid for the HFmrEF-improved cluster was farthest in distance from the HFrEF at 7.7 U and closer to the centroid for the are HFrEF cluster at 5.1 U, suggesting that there was greater overlap of the proteomic profiles for HFmrEF-improved patients and HFpEF patients than for HFmrEF-improved and HFrEF patients.

**TABLE 1 Demographics of Patients With HFrEF, HFmrEF, and HFpEF**

| | Overall (N = 173) | HFrEF (n = 47) | HFmrEF (n = 83) | HFpEF (n = 43) | p Value |
|---|---|---|---|---|---|
| Age, yrs | 55 ± 12.55 | 56 ± 12.59 | 54 ± 11.97 | 56 ± 13.70 | 0.61 |
| White | 127 (73) | 35 (74) | 62 (75) | 30 (70) | 0.83 |
| Black | 46 (27) | 12 (26) | 21 (25) | 13 (30) | 0.83 |
| Male | 100 (58) | 27 (57) | 49 (59) | 24 (56) | 0.94 |
| Diabetes | 44 (25) | 12 (26) | 23 (28) | 9 (21) | 0.71 |
| High blood pressure | 96 (55) | 26 (55) | 42 (51) | 28 (65) | 0.30 |
| CAD | 41 (24) | 21 (45) | 14 (17) | 6 (14) | **<0.01** |
| Emphysema (COPD) | 25 (14) | 5 (11) | 10 (12) | 10 (23) | 0.16 |
| PVD | 1 (1) | 1 (2) | 0 (0) | 0 (0) | 0.26 |
| CVA/TIA | 16 (9) | 5 (11) | 4 (5) | 7 (16) | 0.10 |
| High cholesterol (HLD) | 75 (43) | 28 (60) | 28 (34) | 19 (44) | **0.02** |
| OSA | 47 (27) | 15 (32) | 18 (22) | 14 (33) | 0.30 |
| AF/atrial flutter | 9 (5) | 3 (6) | 1 (1) | 5 (12) | **0.04** |
| ICD/CRT | 86 (50) | 35 (74) | 41 (49) | 10 (23) | **<0.01** |
| Heart rate, beats/min | 76 ± 13.39 | 82 ± 13.98 | 74 ± 12.30 | 74 ± 13.22 | **<0.01** |
| SBP, mm Hg | 118 ± 19.79 | 111 ± 19.77 | 120 ± 16.29 | 123 ± 23.77 | **0.01** |
| DBP, mm Hg | 72 ± 11.12 | 69 ± 11.28 | 75 ± 8.87 | 72 ± 13.90 | **0.03** |
| Mean arterial pressure, mm Hg | 88 ± 13.08 | 83 ± 13.62 | 90 ± 10.54 | 89 ± 15.74 | **0.02** |
| Creatinine, mg/dl | 1.15 (0.86-1.42) | 1.22 (0.94-1.53) | 1.09 (0.89-1.37) | 0.98 (0.81-1.49) | 0.18 |
| Sodium, mmol/l | 139 ± 3.36 | 138 ± 3.27 | 140 ± 3.19 | 140 ± 3.66 | 0.12 |
| eGFR, ml/min/1.73 $m^2$ | 72 ± 28.96 | 65 ± 30.64 | 74 ± 25.67 | 74 ± 32.56 | 0.23 |
| ACC/AHA stage | | | | | **<0.01** |
| B | 5 (3) | 0 (0) | 2 (2) | 3 (7) | 0.14 |
| C | 159 (92) | 39 (83) | 80 (96) | 40 (93) | **0.02** |
| D | 9 (5) | 8 (17) | 1 (1) | 0 (0) | **<0.01** |
| NYHA functional class | | | | | **<0.01** |
| I | 24 (14) | 1 (2) | 16 (19) | 7 (16) | |
| II | 96 (55) | 25 (53) | 50 (60) | 21 (49) | |
| III | 40 (23) | 13 (28) | 14 (17) | 13 (30) | |
| IV | 13 (8) | 8 (17) | 3 (4) | 2 (5) | |
| Beta-blockers | 149 (86) | 38 (81) | 75 (90) | 36 (84) | 0.28 |
| ACE inhibitor | 115 (66) | 26 (55) | 63 (76) | 26 (60) | **0.04** |
| ARB | 33 (19) | 13 (28) | 11 (13) | 9 (21) | 0.13 |
| Diuretics | 137 (79) | 37 (79) | 70 (84) | 30 (70) | 0.16 |
| Digoxin | 54 (31) | 18 (38) | 28 (34) | 8 (19) | 0.10 |
| CCB | 25 (14) | 3 (6) | 11 (13) | 11 (26) | **0.03** |
| Nitrates | 48 (28) | 17 (36) | 18 (22) | 13 (30) | 0.19 |
| Anti-HLD | 95 (55) | 33 (70) | 40 (48) | 22 (51) | **0.04** |
| Hydralazine | 31 (18) | 13 (28) | 11 (13) | 7 (16) | 0.12 |
| Antiplatelet | 112 (65) | 33 (70) | 54 (65) | 25 (58) | 0.49 |
| Warfarin | 51 (29) | 21 (45) | 21 (25) | 9 (21) | **0.02** |
| MRAs | 63 (36) | 20 (43) | 31 (37) | 12 (28) | 0.35 |

Values are mean ± SD, n (%) of observations, or median (interquartile range). **Bold** p values are statistically significant.

ACC/AHA = American College of Cardiology/American Heart Association; ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; AF = atrial fibrillation; CAD = coronary artery disease; CCB = calcium-channel blocker; COPD = chronic obstructive pulmonary disease; CVA = cerebrovascular accident; DBP = diastolic blood pressure; eGFR = estimated glomerular filtration rate; HFmrEF = heart failure with mid-range ejection fraction; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; HLD = hyperlipidemia; ICD/CRT = implantable cardioverter defibrillator/cardiac resynchronization therapy; MRAs = mineralocorticoid receptor antagonists; NYHA = New York Heart Association; OSA = obstructive sleep apnea; PVD = peripheral vascular disease; SBP = systolic blood pressure; TIA = transient ischemic attack.

Comparative heat maps were also used to visualize the differences and similarities in the proteomic signatures for subjects with HFrEF, HFmrEF, and HFpEF. As illustrated in Figure 2, 120 proteins were differentially expressed (62 increased/58 decreased) between patients with HFrEF versus patients with HFpEF, 183 were differentially expressed (96 increased/58 decreased) between patients with HFrEF versus HFmrEF, and 54 proteins were differentially expressed (31 increased, 23 decreased) between patients with HFmrEF versus HFpEF. These heat map data are internally consistent with the analyses of centroid clusters (Figure 1A). Viewed together, these data suggest that the molecular

**TABLE 2 Etiologies of HF for the Patient Cohorts**

| Etiology | Total (N = 173) | HFrEF (n = 47) | HFmrEF-Improved (n = 59) | HFmrEF-Unimproved (n = 24) | HFpEF (n = 43) | p Value |
|---|---|---|---|---|---|---|
| Ischemic (CAD) | 41 (24) | 21 (45) | 9 (15) | 5 (21) | 6 (14) | 0.57 |
| Nonischemic | 132 (76) | 26 (55) | 50 (85) | 19 (79) | 37 (86) | |
| Idiopathic | 96 (56) | 22 (47) | 46 (78) | 11 (46) | 17 (40) | <0.01 |
| Amyloid | 1 (1) | | | 1 (5) | | |
| Congenital | 6 (4) | | | 1 (5) | 5 (12) | |
| Hypertrophic | 5 (3) | 1 (3) | | 2 (9) | 2 (5) | |
| Peripartum | 7 (5) | 2 (5) | 1 (2) | 2 (9) | 2 (5) | |
| Restrictive | 1 (1) | | | | 1 (3) | |
| Valvular | 2 (2) | 1 (3) | | 1 (5) | | |
| Other | 13 (8) | | 2 (4) | 1 (5) | 10 (24) | |
| Mixed | 1 (1) | | 1 (2) | | | |

Values are n (%). The p values shown are for the difference in frequency between HFmrEF-improved and HFmrEF-unimproved subgroups.

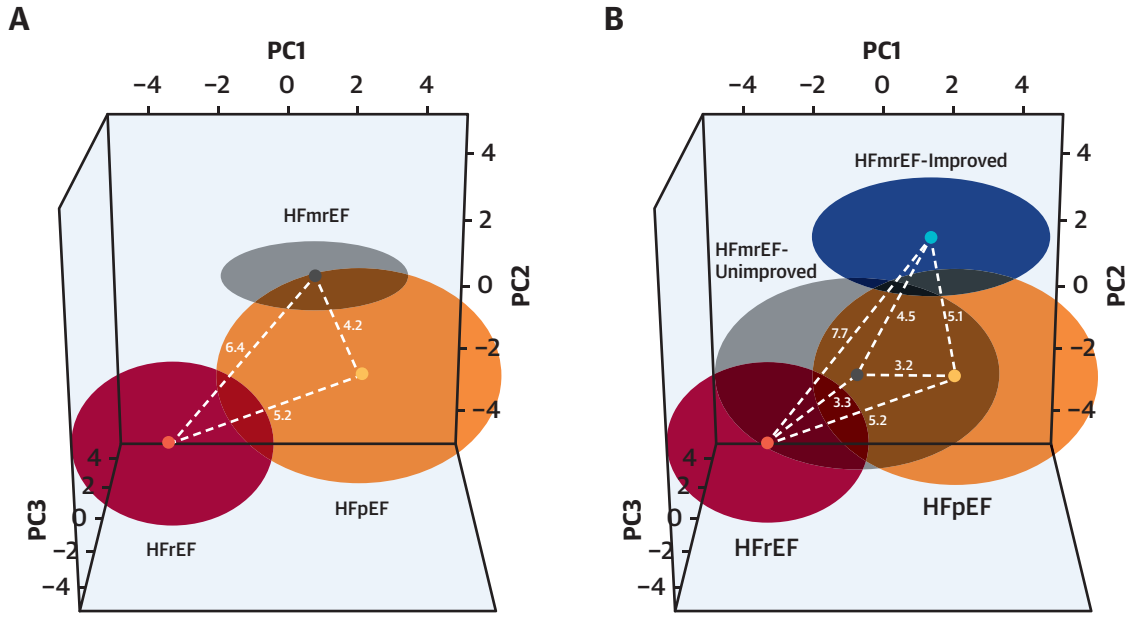
EF = ejection fraction; HF = heart failure; other abbreviations as in Table 1.

similarity, as represented by plasma protein levels, is greatest for HFmrEF and HFpEF patients and least for HFmrEF and HFrEF patients.

Given that the PCA analysis of HFmrEF-improved and HFmrEF-unimproved cohorts revealed minimal overlap of their protein profiles, additional heat maps were generated for the protein levels in both groups, which were then compared with HFrEF and HFpEF patients. Figures 3A and 3B demonstrate that the greatest differences in relative protein levels (ranked from greatest to least) were observed for HFmrEF-improved versus HFrEF patients (226 proteins [114 increased/112 decreased]) > HFmrEF-improved versus HFpEF patients (81 proteins [52 increased/ 21 decreased]) > HFmrEF-unimproved versus HFrEF (35 proteins [18 increased/17 decreased]) ≈ HFmrEF-unimproved versus HFpEF patients (26 proteins [16 increased/10 decreased]). These data are fully consistent with the analyses of the distances between the centroid clusters shown in Figure 1B, and suggest that the similarity of the plasma protein levels are greatest for HFmrEF-unimproved and HFrEF patients and least for HFmrEF-improved and HFrEF patients.

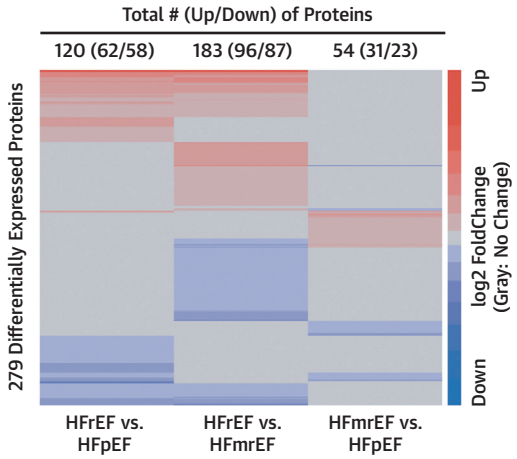
To determine whether the blood proteome reflected proteins that were expressed in the heart, we compared the 280 proteins that were differentially expressed in the HFrEF, HFmrEF, and the HFpEF patients with those known in the heart-proteome. Of the 280 proteins that were differentially expressed among the different LVEF classifications in the

**FIGURE 1 3-Dimensional PCA Analysis of the Spatial Relationship of the Serum Proteome Profiles of Different EF Groups**




The serum proteome of patients with different left ventricular ejection fraction (LVEF) at the time of enrollment is depicted on a 3-dimensional principal components analysis (PCA) plot. **(A)** The 3 major ejection fraction groups are identified, and **(B)** 4 different groups are identified because the heart failure with a midrange LVEF (HFmrEF) group is divided into HFmrEF-improved and HFmrEF-unimproved. A centroid (**small round dot within an ellipse**) was calculated based on the PCA vectors for each group and the spatial distance between centroids was calculated. HFpEF = HF with preserved ejection fraction; HFrEF = HF with reduced ejection fraction.

**FIGURE 2 Heat Maps of Differentially Expressed Serum Proteins for Patients With HFrEF, HFmrEF, and HFpEF**

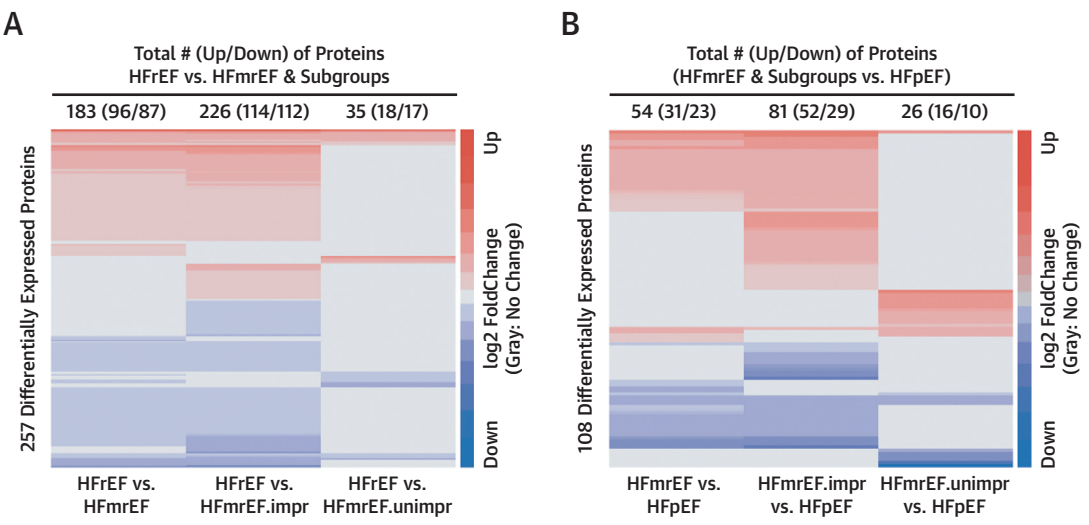



We clustered proteins with the Euclidean distance and the Ward clustering algorithm. The proteins nondifferentially expressed between the 2 groups of interest are shown in **gray** to highlight the differences and similarities between groups. Key: **red** = increased expression; **blue** = decreased relative expression. Abbreviations as in Figure 1.

present study, 6 proteins were specific to the heart, 139 were expressed in the heart as well as other tissues, and 135 were either not expressed in the heart, or were expressed with low tissue specificity. Thus, approximately 51% of the circulating proteins that were detected in the present study were either specific to the heart, or the heart as well as other tissues (Supplemental Table 2).

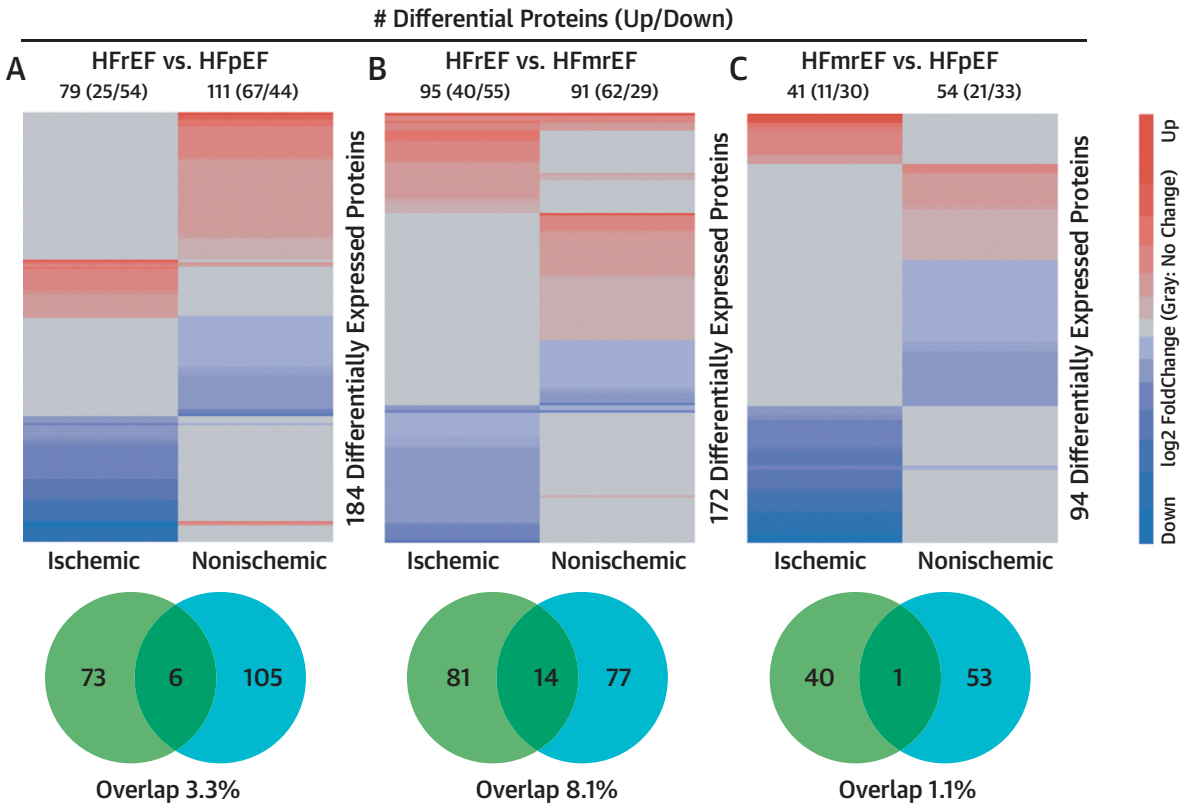
**EFFECT OF HF ETIOLOGY ON PROTEIN LEVELS IN LVEF SUBGROUPS.** The covariate analysis shown in Supplemental Figure 2 revealed that the etiology of heart failure contributed approximately 6% to 14% of the variability of the signal values for the plasma proteomic profiles. Given this, an analysis was completed to determine if the differential protein expression in patients with ischemic and nonischemic HF stratified by LVEF. Figure 4 shows that the greatest differences in protein expression levels for the pairwise comparison of ischemic versus nonischemic HF etiology were (in rank order) for HFrEF versus HFpEF (184 proteins) ≈ HFrEF versus HFmrEF (172 proteins) > HFmrEF versus HFpEF (94 proteins). Intriguingly, the pairwise comparisons of differentially expressed proteins revealed that there was surprisingly little overlap of the proteins between

**FIGURE 3 Heat Maps of Differential Representation of Serum Proteins Comparing HFrEF and HFpEF Versus HFmrEF-Improved and HFmrEF-Unimproved**




We clustered proteins with the Euclidean distance and the Ward clustering algorithm. The proteins nondifferentially expressed between the 2 groups of interest are shown in **gray** to highlight the differences and similarities between groups. **(A)** Comparisons between HFmrEF and its subgroups versus HFrEF. **(B)** Comparisons between HFmrEF and its subgroups versus HFpEF. Key: **red** = increased expression; **blue** = decreased relative expression. Abbreviations as in Figure 1.

**FIGURE 4** Heat Maps and Venn Diagrams of Serum Proteins Differentially Expressed Across Major LVEF Subgroups Categorized by HF Etiology




Heat maps of proteins differentially expressed in the pairwise comparisons for each of the 3 major ejection fraction (EF) groups stratified by ischemic versus nonischemic etiology. **(A)** HFrEF versus HFpEF, **(B)** HFmrEF versus HFpEF, and **(C)** HFmrEF versus HFpEF. Proteins were hierarchically clustered with the Euclidean distance and the Ward cluster method. The proteins nondifferentially expressed between the 2 groups of interest are shown in **gray** to highlight the differences and similarities between groups. Key: **red** = increased expression; **blue** = decreased relative expression. HF = heart failure; LVEF = left ventricular ejection fraction; other abbreviations as in Figure 1.

patients with an ischemic or nonischemic etiology of HF, regardless of the LVEF stratification. The Venn diagrams in Figure 4 show that the overlap was greatest for the comparison of HFrEF versus HFmrEF (8.1%), followed by HFrEF versus HFpEF (3.3%), and was least for HFmrEF versus HFpEF (1.1%). The sample size was considered too small to investigate the effects of the etiology of HF within the HFmrEF subgroups, so this analysis was not performed.

Given the differences in plasma proteome observed between patients with ischemic and nonischemic cardiomyopathy, we asked whether the differences in serum proteome profile observed among patients with HFrEF, HFmrEF-improved, HFmrEF-unimproved, and HFpEF were the result of differences between ischemic and nonischemic HF. Accordingly, we repeated the analysis shown in Figure 1 by determining the centroids for the ischemic and nonischemic patients in the HFrEF, HFmrEF (HFmrEF-improved and HFmrEF-unimproved), and HFpEF subgroups. As shown in Supplemental Figure 3, the centroids for ischemic and nonischemic patients were in close proximity, suggesting that differences in proteomic profiles for ischemic versus nonischemic patients did not explain the differences in the proteome between the different LVEF classifications.

**COMPREHENSIVE BIOLOGICAL THEME ANALYSIS.** A biological theme analysis (pathways, processes, cellular signaling) was performed using the CompBio platform to evaluate circulating proteins that were differentially expressed in HFrEF, HFmrEF, and

HFpEF patients. An example of an annotated output from the specific comparison of HFrEF versus HFmrEF is illustrated in Supplemental Figure 4, with a summarization of themes from all comparisons shown in Supplemental Figures 5A to 5C. Substantial heterogeneity was observed with respect to the categories of enriched proteins for each of the LVEF comparisons. The top 5 thematic biological clusters that were differentially expressed between HFrEF and HFpEF patients were related to increased WNT/IGF signaling, increased TDGF/morphogenic regulation, increased BMP/morphogenic regulation, increased VEGF-A/angiogenesis, and decreased JAK/STAT signal transduction. The 5 most prominent themes for the differences between HFrEF and HFmrEF patients were related to increased IGF/IGBP signaling, increased MMP activity, increased chemokine induction, increased VEGF-A/angiogenesis, and increased follistatin myogenic regulation. In contrast, the top 5 thematic biological differences between HFmrEF and HFpEF patients were related to increased lymphotoxin signaling, increased LPS-TLR signaling, increased complement/chemoattractant signaling, and decreased cathepsin lysosomal activity. Data for key proteins associated with biological themes of interest illustrating the differential expression patterns across the 3 cohorts are shown in Supplemental Figure 5D.

To provide a simple format for visualizing the differences in biological themes between the LVEF groups, the theme lists provided in Supplemental Figures 5A to 5C were used to generate a pseudo heat map of the most salient biological themes that were differently expressed among the HFrEF, HFmrEF, and HFpEF cohorts (**Figure 5**). These themes were further grouped into broader biological categories to facilitate comparisons between groups. Within the HFrEF cohort, 5 categories contained themes that were differentially expressed as compared with both the HFmrEF and HFpEF cohorts. The HFrEF increased expression categories included growth factor signaling, inflammation, neurotrophic signaling, remodeling/hypertrophy and angiogenesis (VEGF-A related), whereas decreased-expression themes included those related to coagulation and myeloproliferation ("other"). Biological themes differentially expressed in HFmrEF patients as compared to HFrEF and HFpEF patients, included decreased inflammation and increased signal transduction. Finally, themes expressed differently in HFpEF patients as compared with HFrEF and HFmrEF patients, included growth factor signaling, inflammation, and angiogenesis (VEGF-C related). Viewed together, this bioinformatics analysis suggests that HFrEF, HFmrEF, and HFpEF have proteomic signatures that reflect distinct pathophysiologies.

We also created a pseudo heat map to delineate the salient biological themes that were differently expressed between ischemic and nonischemic patients with HF. As shown in Supplemental Figure 6, biological themes related to increased growth factor signaling were numerically more predominant in ischemic versus nonischemic HF, whereas the number of themes related to decreased inflammation were numerically less prominent in ischemic versus nonischemic HF. However, to our surprise, there were no striking differences in the biological themes between ischemic versus nonischemic HF.

## DISCUSSION

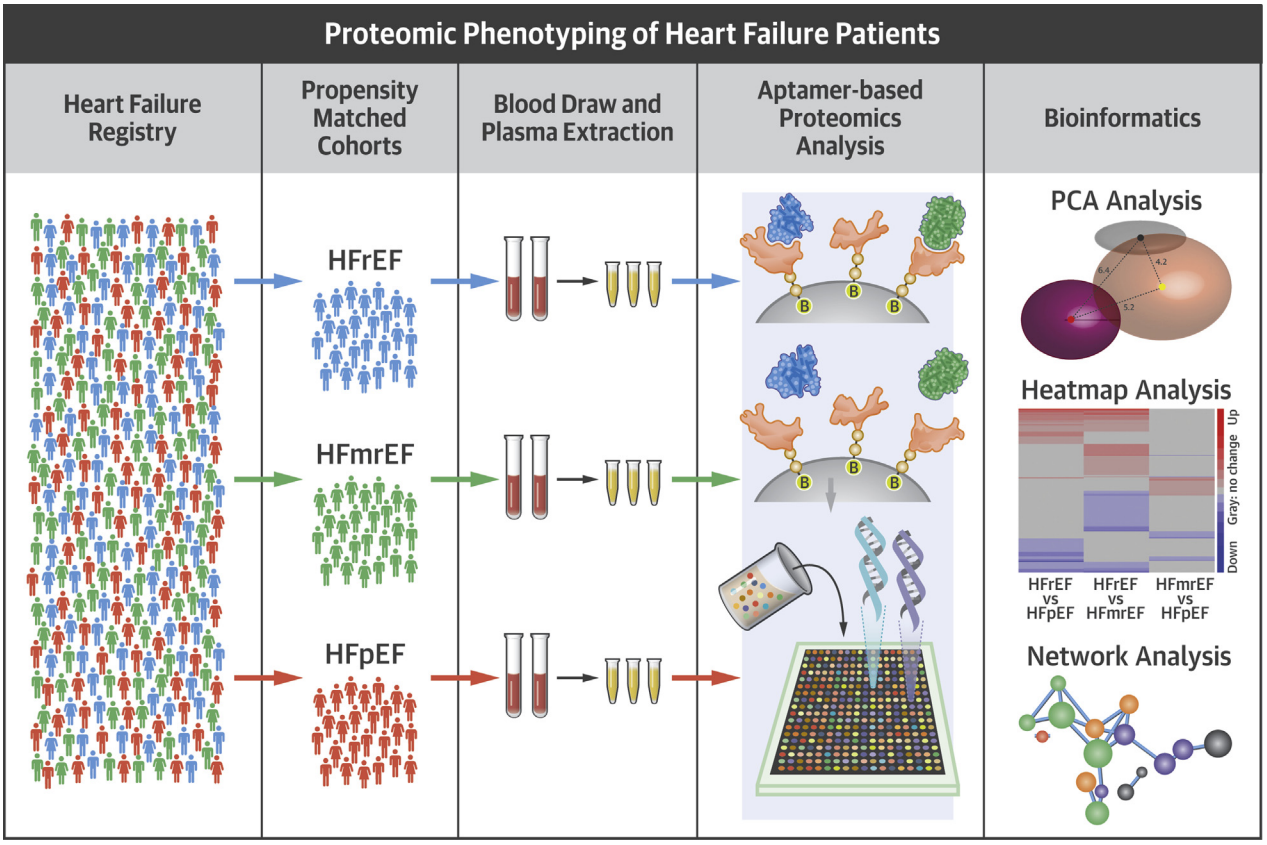
The results of this study, in which we used a high-content proteomics assay to analyze the blood proteome of patients with HF stratified by their LVEF, show that although changes in LVEF capture a small portion of the proteome variability across patients with HF, patients with HFrEF, HFmrEF, and HFpEF have specific proteomic signatures that reflect distinct pathobiologies (**Central Illustration**). Accordingly, the proteomic signatures for patients with HFrEF and HFpEF were different (**Figures 1, 2, and 5**), consistent with the prevailing view that these 2 different classifications of HF have different underlying pathophysiologies and respond differently to medical therapies. Bioinformatics analysis revealed that there were biological themes that were unique to HFrEF patients when compared with HFpEF and HFmrEF patients, including increased growth factor signaling, increased extracellular matrix remodeling, increased angiogenesis (**Figure 5**), reflecting the greater degree of LV remodeling (dilation) and LV systolic dysfunction in patients with HFrEF. Intriguingly, there was an increase in circulating proteins related to innate immunity and a decrease in the expression of proteins related to humoral immunity in HFrEF patients compared with HFpEF and HFmrEF patients. The biological themes that were unique to HFpEF patients when compared with HFrEF and HFmrEF patients were enriched for proteins reflecting growth factor signaling, increased humoral immunity, and increased angiogenesis (**Figure 5**).

Patients with midrange LVEF represent a heterogeneous patient population composed of a large proportion of patients with HFrEF whose LVEF partially recovered on evidence-based medical therapies, and a smaller proportion of patients with HFpEF whose LVEF declined. Remarkably, the plasma proteome of patients with HFmrEF was

**FIGURE 5** Pseudo Heat Map of Differentially Expressed Proteins for Patients With HFrEF, HFmrEF, and HFpEF


A pseudo heat map was constructed from the comparisons of differentially expressed proteins illustrated in Supplemental Figures 5A to 5C for patients with HFrEF, HFmrEF, and HFpEF. Related biological themes from the list of differentially expressed proteins in Supplemental Figures 5A to 5C were grouped into higher-level biological groupings to permit comparisons between the various heart failure (HF) subgroups. Key: **red** = increased expression; **blue** = decreased relative expression. Abbreviations as in **Figure 1**.

**CENTRAL ILLUSTRATION** **Patients With Heart Failure Stratified by Their Left Ventricular Ejection Fraction Have Distinct Proteomic Signatures**




**Adamo, L. et al. J Am Coll Cardiol. 2020;76(17):1982–94.**

A cohort of age- and sex-matched patients was randomly selected from the Washington University Heart Failure Registry. Patients were stratified into 3 heart failure (HF) cohorts and were identified based on their left ventricular ejection fraction (LVEF): HF with reduced ejection fraction (HFrEF) (LVEF <40%), HF with a preserved ejection fraction (HFpEF) (LVEF >50%), and HF with a midrange ejection fraction (HFmrEF) (LVEF 40% to 50%). A high-content aptamer-based proteomics assay was used to analyze the blood proteome of the patients in each cohort. Bioinformatics analysis showed that patients with HFrEF, HFmrEF, and HFpEF have specific proteomic signatures that reflect distinct pathobiologies. A high-content proteomics assay was used to analyze the blood proteome of patients with HFrEF (LVEF <40%), HFmrEF (LVEF 40% to 50%), and HFpEF (LVEF >50%). Patients with HFrEF, HFmrEF, and HFpEF had unique variations in circulating proteins that reflected distinct biological pathophysiologies. Bioinformatics analysis revealed biological themes that were unique to HFrEF, HFpEF, and HFmrEF patients, suggesting that it may be possible to use proteomics assays to more accurately predict clinical phenotypes of patients with HF.

distinct from the plasma proteome of patients with HFrEF (Figures 1A and 2), and partially overlapped that of patients with HFpEF, in agreement with prior clinical observations that patients with HFmrEF have a functional capacity and clinical outcomes that are similar to that of patients with HFpEF (6,9). Bioinformatics analysis suggested that the circulating proteins in patients with HFmrEF were enriched for themes reflecting changes in adaptive immune responses and increased signal transduction (Figure 5). When we further subclassified the patients with HFmrEF into the HFmrEF-improved and HFmrEF-unimproved, we observed that the protein signature of HFmrEF-unimproved patients partially overlapped that of HFrEF and HFpEF patients, whereas the proteomic signature for HFmrEF-improved patients was completely distinct from HFrEF patients, and partially overlapped that of patients with HFpEF (Figures 1 and 2). To the extent that levels of proteins in the circulatory system reflect the physiology of a given individual, the observation that the plasma proteomic profile of HFmrEF-improved patients was

distinct from that of HFrEF patients suggests that patients who partially recover their LVEF on evidence-based medical therapies are biologically unique and are different from patients with HFrEF whose LVEF did not recover on medical therapy. The finding that the plasma proteomic profile of HFmrEF-improved patients overlaps that of HFpEF patients is consistent with the concept that recovery of LV function, although associated with improved event-free survival, is still associated with a persistent HF risk and recurrent HF hospitalizations (i.e., "myocardial remission") (6,10).

An unanticipated finding of these studies was the marked difference in the proteomic signatures of patients with ischemic and nonischemic HF. Indeed, there was <10% overlap of the plasma proteomic profiles of ischemic and nonischemic patients, regardless of the LVEF stratification (**Figure 3**). Although it has long been recognized that the clinical outcomes for patients with ischemic and nonischemic HF are different (11). Our data suggest that biological differences in ischemic and nonischemic patients with HF are greater than previously supposed.

### PROFILING THE BLOOD PROTEOME IN HEART FAILURE.

The use of protein biomarkers (e.g., B-type natriuretic peptide [BNP] or N-terminal-proBNP) has become routine in the clinical diagnosis and management of patients with HF (reviewed in [12,13]). The appreciation that single biomarker strategies do not reflect all aspects of the complex pathophysiology of HF has given rise to the use of multi-biomarker testing panels to improve medical care (12-14). However, current multi-biomarker approaches use multiplexed immunoassays that have inherent technical limitations, such as lack of specificity for different protein isoforms, insensitivity to detect lower abundance proteins, and antibody cross-reactivity. Moreover, although multi-maker strategies are able to measure more aspects of the pathophysiology of HF than single biomarkers, multibiomarker strategies have inherent limitations in terms of revealing the complex physiology of HF, insofar as they measure predefined proteins in known pathways, and thus are not able to detect clinically relevant proteins in unrecognized biological pathways.

Although high-throughput, quantitative analysis using mass-spectrometry (MS)-based proteomics of blood, plasma, and urine is an extremely attractive unbiased approach to assess the circulating proteome, the application of untargeted MS has been challenging thus far because of the high dynamic range of protein abundances (5), as well as the lack of reproducible, robust, high-throughput workflows to reliably identify and verify potential biomarkers. As a result, untargeted MS-based proteomic approaches have not been used in HF. Emerging technologies that address these issues include nucleotide-labeled immunoassays and aptamer reagents, which can be automated for efficient multiplexing of thousands of proteins with high throughput. Aptamer proteomics have been applied to large cohorts of patients to identify hundreds of novel protein biomarkers for coronary artery disease (15) and myocardial injury (16). Furthermore, aptamer proteomics have been used alongside ELISA to validate novel HF biomarkers (17). Germane to this discussion, a recent study using aptamer-based proteomics in 43 patients with HF (median LVEF 52% [IQR: 32% to 55%]) and 43 control subjects identified 9 candidate HF biomarkers. Two of these proteins, angiopoietin-2 and thrombospondin-2, were subsequently confirmed in separate validation cohorts as robust biomarkers for detecting acute and preclinical HF (18). Although the scope of this study was not intended to identify specific HF biomarkers within each LVEF classification, we did observe significantly ($p < 0.05$) increased levels of both angiopoietin-2 and thrombospondin-2 in patients with HFrEF when compared to HFmrEF and HFpEF subgroups (Supplemental Figure 5D).

Our findings are in overall agreement with a prior study that measured 92 biomarkers in the plasma of HFrEF, HFmrEF, and HFpEF patients enrolled in the Scottish Cohort of the BIOSTAT-CHF (BIOlogy Study to TAilored Treatment in Chronic Heart Failure) study, which used proximity extension assay technology (Olink Proteomics, Uppsala, Sweden) (18), and concluded that their study provided "biological context for the presence of clearly distinct syndromes" (i.e., HFrEF, HFmrEF, and HFpEF). The results of the present study both confirm and expand on this study, which reported upregulation of biological pathways related to cellular growth, including growth differentiation factor-15 (Supplemental Figure 5) in patients with HFrEF, and increased inflammation in HFpEF (18). Because of the 14-fold broader coverage with the SOMAscan (13,000 proteins vs. 92 proteins) we were able to identify 279 differentially expressed proteins among the HF groups, as opposed the 14 unique proteins in the BIOSTAT-CHF registry. The broader coverage allowed us to identify novel biological themes in HFmrEF (decreased inflammation and increased signal transduction) and HFpEF (growth factor signaling, angiogenesis) that were not detected in the study by Tromp et al. (14), as well as

expand on themes in HFrEF (remodeling/hypertrophy and angiogenesis). We were also able to identify unique differences between HFmrEF-improved and HFmrEF-unimproved, which was not explored in the baseline samples BIOSTAT-CHF registry, whose samples were obtained (by design) from suboptimally treated patients with HF.

**STUDY LIMITATIONS.** First, although the samples that were used for this study were obtained from a well-phenotyped cohort of patients with HF, the number of patients that were studied is relatively small, drawn from a single center, and because of the use of propensity matching the age of the patients with HFpEF is younger than traditional HFpEF cohorts. For these reasons, the results of this study should be regarded as provisional, pending validation in separate cohorts of patients with HF from different centers. Second, only 29% of the patients with HFmrEF were in the HFmrEF-unimproved subgroup. The imbalance in sample size between the HFmrEF-unimproved and HFmrEF-improved groups has the potential to introduce a bias in the differential protein expression analysis reported. Given that the subgroups analyzed were not perfectly matched in terms of medical therapy, we cannot exclude that the differences in serum proteome profile were, at least in part, the result of differences in medical treatment among the different groups.

## CONCLUSIONS

High-throughput proteomic approaches allow for the detection of thousands of circulating proteins in real time, and thus have the potential to provide a broader and more inclusive mechanistic understanding of the dysregulation of the molecular pathways in HF than is currently possible using single or multi-biomarker panels. The results of this study demonstrate that it is feasible to use multiplex aptamer proteomics to identify distinct proteomic signatures for patients with HFrEF, HFmrEF, and HFpEF, as well as to identify important differences in the proteome of ischemic and nonischemic patients with HF. The current work is hypothesis generating, and it should be viewed as a first step toward potential deeper phenotyping of patients with HF that goes beyond the conventional assessment of LVEF. Our findings will require verification in separate and larger cohorts of patients with HF. Nonetheless, the results of this study suggest that high-content proteomics can be used to identify important biological differences between patients with HF that are subclassified according to conventional LVEF classifications (**Figure 5**). Although the exact clinical applicability of these findings remains to be determined, there are several potential applications that can be envisioned. First, given that the LVEF cutoffs that have been used were assigned arbitrarily based on the need to identify enriched cohorts of patients for clinical trials, it may be possible to use proteomic approaches to better define conventional LVEF cutoffs. For example, the proper LVEF cutoff to define HFpEF is unknown, with values ranging from >45%, >50%, and >60% proposed by different groups. Indeed, the recent results of the PARAGON (A Multicenter, Randomized, Double-blind, Parallel Group, Active-controlled Study to Evaluate the Efficacy and Safety of LCZ696 Compared to Valsartan, on Morbidity and Mortality in Heart Failure Patients [NYHA Class II-IV] With Preserved Ejection Fraction) trial (19), wherein patients with HFpEF with an LVEF of 45% to 50% appeared to respond differently to sacubitril/valsartan than HFpEF with an LVEF ≥50%, suggests that there are biologically meaningful differences among patients with HFpEF. It may be possible to use high-content proteomic approaches to better identify these patients. Second, our findings with respect to the biological differences of HFmrEF-improved patients, suggest that it may be possible to use proteomic strategies to distinguish HFrEF patients with a recovered LVEF from patients with HFpEF, and perhaps identify those patients who can be weaned from medical therapy and those who should remain on evidence-based medical therapies. Moreover, the results of this study raise the exciting possibility that it may be possible in the not too distant future to combine the longitudinal assessment of high-content proteomics assays with machine-learning algorithms to predict the response of patients with HF to medical therapies, as well as predict changes in the health care status of patients with HF. Finally, it remains to be determined whether the variations in proteomic profile that we observed across groups were a cause or effect of the different HF phenotypes. Whether proteomics-based approaches will be superior to single or multi-biomarker panel approaches for personalizing the care of patients with HF is an important question that will require a deeper understanding of the limitations, costs, and the clinical applicability of these different approaches.

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## PERSPECTIVES

**COMPETENCY IN MEDICAL KNOWLEDGE:** There are variations in circulating proteins in patients with HF across a range of LVEF related to differing pathophysiology that is not entirely captured by categorizations as HF with preserved, reduced, or minimally reduced EF (HFpEF, HFrEF, or HFmrEF). Serum proteomic profiles differ in patients with ischemic versus nonischemic cardiomyopathies and in those with static versus improved EF.

**TRANSLATIONAL OUTLOOK:** Integration of left ventricular EF with multiplex proteomics assays holds the potential to improve identification of clinical phenotypes of patients with HF.

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**KEY WORDS** heart failure, left ventricular ejection fraction, proteomics

**APPENDIX** For supplemental methods, tables, and figures, please see the online version of this paper.