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European Journal of Heart Failure (2021)
doi:10.1002/ejhf.2291

RESEARCH ARTICLE

Proteomics to improve phenotyping in obese patients with heart failure with preserved ejection fraction

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Received 23 March 2021; revised 28 June 2021; accepted 2 July 2021

Aims Recent evidence points towards a distinct obese phenotype among patients with heart failure with preserved ejection fraction (HFpEF). We aimed to identify differentially expressed circulating biomarkers in obese HFpEF patients and link them to disease severity and outcomes.

Methods and results From the LIFE-Heart study, 999 patients with HFpEF and 999 patients without heart failure (no-HF) were selected and 92 circulating serum biomarkers were measured using a proximity extension assay. Elevation of identified biomarkers was validated in 220 patients from the Aldo-DHF trial with diagnosed HFpEF. HFpEF patients were older and had more comorbidities including coronary artery disease and type 2 diabetes as compared to no-HF patients ($P < 0.05$ for all). After adjusting for covariates, adrenomedullin (ADM), galectin-9 (Gal-9), thrombospondin-2 (THBS-2), CD4, and tumour necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) were significantly higher in obese HFpEF patients [body mass index (BMI) $\geq$30 kg/m^2, $n = 464$] as compared to lean HFpEF (BMI <30 kg/m^2, $n = 535$) and obese no-HF patients (BMI $\geq$30 kg/m^2, $n = 387$) ($P < 0.001$ for both); these findings were verified in the Aldo-DHF validation cohort ($P < 0.001$). Except for CD4 these proteins were associated with increased estimates of left atrial pressure in a linear fashion. Importantly, ADM and CD4 were associated with increased mortality in obese HFpEF patients after adjusting for covariates.

Conclusion Obese HFpEF patients exhibit higher circulating biomarkers of volume expansion (ADM), myocardial fibrosis (THBS-2) and systemic inflammation (Gal-9, CD4) compared to obese non-HFpEF or lean HFpEF patients. These findings support the clinical definition of a distinct obese HFpEF phenotype and might merit further investigation.

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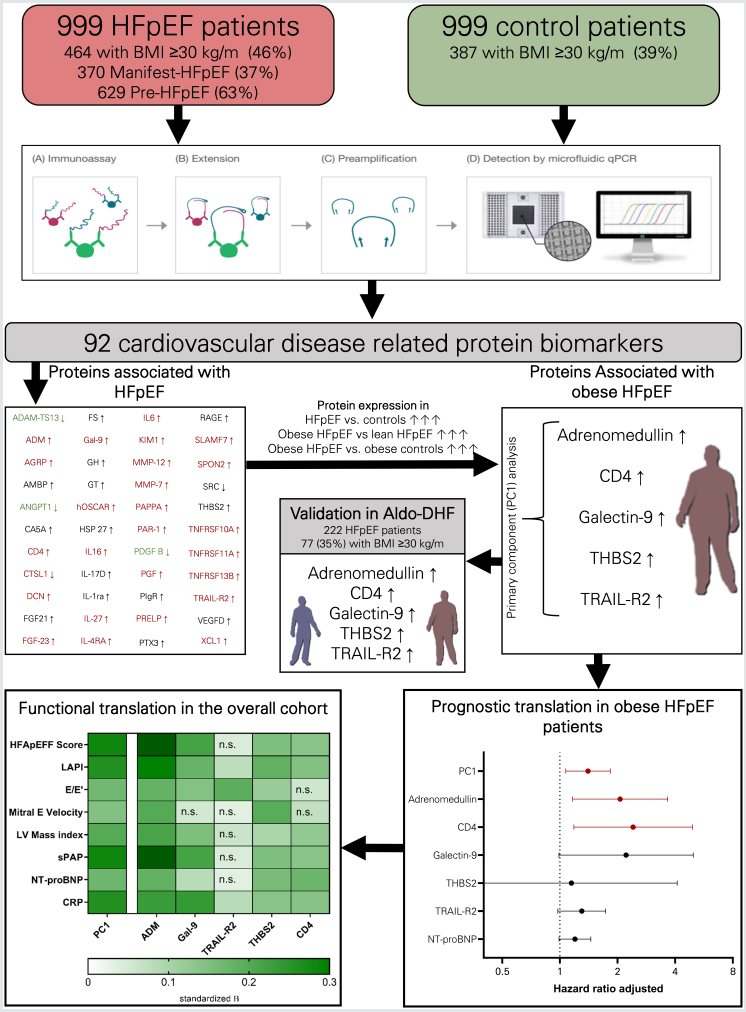
Graphical Abstract



Exploration of 92 proteins from the Olink Cardiovascular II panel and their association with obese heart failure with preserved ejection fraction (HFpEF). Proteins that were different in HFpEF in general as compared to no-heart failure (HF) controls are displayed in the upper left box with an arrow indicating their expression in HFpEF vs. no-HF patients (↑ meaning increase, ↓ meaning decrease). Their association with all-cause mortality is indicated by their colour with red meaning increased mortality and green meaning decreased mortality with increasing serum levels, while black indicates no association with mortality. Proteins with higher serum levels in obese HFpEF patients after adjustment for covariates (age, sex, presence of diabetes, creatinine, presence of coronary artery disease, white blood cell count and haematocrit) are displayed in the upper right box. Their prognostic translation in all-cause mortality in obese HFpEF patients and their functional translation in the overall cohort are depicted in the lower boxes. Hazard ratios in the obese HFpEF group were adjusted for age, sex, renal function, the presence of diabetes and the presence of coronary artery disease. Hazard ratios for the overall HFpEF group were adjusted for age, sex, renal function smoking status, systolic blood pressure at admission, total cholesterol, presence of diabetes, C-reactive protein (CRP), N-terminal pro brain natriuretic peptide (NT-proBNP) and presence of coronary artery disease. BMI, body mass index; LAPI, left atrial anteroposterior diameter index; LV, left ventricle; sPAP, systolic pulmonary artery pressure. For protein abbreviations see online supplementary *Table S3*.

Keywords Heart failure with preserved ejection fraction • Obesity • Fibrosis • Inflammation • Proteomics • Biomarker

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Introduction

A relevant fraction of patients hospitalized for heart failure (HF) show a preserved left ventricular ejection fraction (LVEF) and mortality among these is high.[1,2] Up to now, successful therapeutic approaches are limited to treatment with mineralocorticoid receptor antagonists and angiotensin receptor–neprilysin inhibitors in certain subgroups of HF with preserved ejection fraction (HFpEF) patients.[3,4] Therefore, identifying further specific HFpEF subgroups is crucial to discover therapies that will improve outcomes in future HFpEF treatment strategies. Obesity often mimics clinical and pathophysiological features of HF (e.g. exertional dyspnoea) and has been associated with early development of HFpEF in the young,[5] yet the pathomechanistic understanding of this clinically easily identifiable HFpEF phenotype is poor.[6]

Identifying circulating biomarkers uniquely expressed in patients with HFpEF and obesity might support the prediction of HF disease courses, disease monitoring and identification of previously unrecognized pathophysiological pathways.[7] Studies investigating the protein profile of patients with obesity compared to lean HFpEF phenotypes revealed differences in the expression of parameters for inflammatory activity[8,9] such as the tumour necrosis factor (TNF) pathway.[9] However, as those studies did not include patients with obesity without HF, it remains unclear whether the increased inflammatory state is attributable to obesity alone[10] rather than a specific obese HFpEF phenotype.

We therefore aimed to investigate whether there is evidence for a distinct protein profile in patients with obesity with or without HFpEF as compared to lean HFpEF patients and obese patients without HFpEF and whether such a profile might translate into prognostic value regarding HFpEF severity and premature mortality.

Methods

Study cohort

Within the observational LIFE-Heart cohort,[11] patients with HFpEF and control patients were identified according to current guideline recommendations of the European Society of Cardiology (ESC).[12] Between 2006–2014, patients were consecutively included into the LIFE-Heart study at our tertiary care centre and were comprehensively evaluated including invasive coronary angiography, echocardiography and laboratory testing.[13] HFpEF in patients was defined by a LVEF ≥50%, elevated N-terminal pro brain natriuretic peptide (NT-proBNP, ≥125 ng/L), and evidence of structural heart disease defined by either diastolic dysfunction (E/E′ ≥13), left ventricular hypertrophy (≥115 g/m^2 in men; ≥95 g/m^2 in women) and/or left atrial (LA) dilatation (≥34 mL/m^2 or anteroposterior diameter ≥23 mm/m^2). The HFpEF group included patients with a 'manifest HFpEF' diagnosis requiring patients to report signs and/or symptoms of HF and patients with 'pre-HFpEF' where signs of cardiac structural alterations and elevated natriuretic peptides were present in the absence of HF specific symptoms and signs. If not otherwise stated, 'HFpEF' patients included both pre- and manifest HFpEF patients. The control group was comprised of patients without overt HF characterized by a LVEF ≥50% and NT-pro-BP concentrations <125 ng/L. Obesity was defined by a body mass index (BMI) ≥30 kg/m^2. Plasma volume was estimated by (1-haematocrit) × [a + (b × weight in kg)] where a = 1530 for men and 864 for women, and b = 41 for men and 47.9 for women, respectively.[14] Exclusion criteria were the presence of acute coronary syndrome, LVEF <50%, relevant valvular disease (more than moderate aortic stenosis – regurgitation or mitral regurgitation; more than mild mitral stenosis), missing classification data, age <40 years, and inconclusive classification. Survival status was verified yearly via hospital records and inquiries at central residence registers. Out of the available patients, 999 HFpEF patients were randomly sampled and analysed, 999 control cases for comparison were selected to roughly fit age, sex and BMI specifics of the HFpEF cohort as outlined in the supplemental material in more detail. The study meets the ethical standards of the Declaration of Helsinki. It has been approved by the local ethics committee and is registered with ClinicalTrials.gov (NCT00497887). Written informed consent was obtained from all participants enrolled in the study.

Validation cohort

To validate identified proteins from the derivation cohort, patients from the randomized prospective Aldosterone Receptor Blockade in Diastolic Heart Failure (Aldo-DHF) study were included. The study design and main findings were reported previously.[15] In brief, between 2007 and 2012, 422 patients with chronic New York Heart Association (NYHA) class II or III, preserved LVEF of ≥50%, and evidence of diastolic dysfunction were randomly assigned to receive either 25 mg of spironolactone or placebo for 12 months. Patients were classified as HFpEF when they fulfilled the ESC criteria for HFpEF as outlined in detail above.[15] Out of all patients included into the Aldo-DHF trial, 220 fulfilled current ESC criteria for the diagnosis of HFpEF and served as the validation cohort.

Biomarker measurement

In each patient, peripheral venous blood samples were drawn immediately after informed consenting in the fasted state. Clinical chemistry was measured on the day of blood sampling using an automated Roche Modular analysis system (Roche Diagnostics, Mannheim, Germany). Multiple aliquots of plasma samples were stored at −80°C or liquid nitrogen for further analysis. In 2019 and 2020, plasma samples from the Aldo-DHF and LIFE-Heart cohorts, respectively, were post-hoc analysed for circulating protein biomarkers using the Olink (Uppsala, Sweden) Cardiovascular II panel. This panel was chosen as it reflects biomarkers proposed to be associated with inflammatory, immuno–cardiovascular interaction, tissue and vascular neogenesis as well as cardio–metabolic interaction. Cases and controls were randomly distributed across plates, and assays were performed in a blinded fashion. For the assay, a proximity extension assay technology was used where 92 oligonucleotide-labelled antibody probe pairs per panel may bind to their respective targets in 1 mL plasma sample.[9,16] When bound, they give rise to new DNA amplicons with each identification barcoding their respective antigens. Quantification of the amplicons was subsequently performed using a Fluidigm BioMark HD real-time polymerase chain reaction (PCR) platform. The PCR platform provides log2-normalized protein expression (NPX) data and an increase of 1 NPX means a doubling in concentration of the specific biomarker. All assay validation data are available on the manufacturer's website (www.olink.com). When levels of any biomarker fell below the limit of detection in ≥10% of the study population, the biomarker was excluded from further analysis (in the LIFE-Heart cohort: fibroblast growth factor 23, 23%; integrin beta 1 binding protein 2, 33%).

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Statistical analysis

Parametric data (Kolmogorov–Smirnov test) are given as their mean and corresponding standard deviation. If data were non-parametric, they are presented as median and corresponding interquartile range (IQR); differences in protein concentrations are also shown as fold changes. Continuous variables were compared with Student's t-test and Mann–Whitney U test where appropriate. Categorical variables were compared using the Fisher's exact test.

Kaplan–Meier analyses were used to compare survival in different subgroups; the log-rank test was used to test for differences. Cox regression analyses were performed to test the prognostic relevance of continuous variables with regard to all-cause mortality; results are presented as hazard ratios (HR) with corresponding 95% confidence interval (CI).

In a first step, we analysed proteins that were associated with pre- and manifest HFpEF. In a next step, we identified proteins that were significantly higher expressed in obese HFpEF patients when compared to lean HFpEF patients as well as obese no-HF patients after adjustment for multiple testing (Bonferroni correction). The association of these proteins with HFpEF and obesity was then adjusted for age, sex, presence of diabetes, creatinine, presence of coronary artery disease, white blood cell count and haematocrit as those variables might influence circulating biomarker levels. Further, the identified differences in protein concentrations were then investigated in the validation cohort, adjusting for the above-mentioned variables.

A primary component analysis was performed to reduce the identified proteins to a single protein score, which could be established for each individual patient. The identified primary component as well as the respective individual proteins and their association with functional HFpEF-related clinical characteristics [HFA-PEFF score, LA anteroposterior diameter index, E/E′; left ventricular mass index, systolic pulmonary artery pressure (sPAP), NT-proBNP, and C-reactive protein (CRP)] were than analysed using linear regression analysis with adjustment for age, sex and renal function.

A two-sided significance level of $\alpha \leq 0.05$ was defined appropriate to indicate statistical significance. In case of multiple testing, a Bonferroni adjustment was performed multiplying the test significance niveau times the number of hypothesis tested. Statistical analyses were performed using the SPSS software (IBM Corp. released 2017, IBM SPSS Statistics for Windows, version 25.0. Armonk, NY, USA).

Results

Patient characteristics of the entire cohort

Overall, 6995 patients were consecutively included in the LIFE-Heart study between 2006 and 2014. After application of the inclusion and exclusion criteria (*Figure 1*), 1048 patients with HFpEF and 1583 control patients were identified. Out of those, 999 patients were sampled per group as outlined in the supplementary material; 370 patients had manifest HFpEF (37%) and 629 pre-HFpEF (63%). Characteristics of excluded patients are displayed in online supplementary *Table S1*. Overall, 464 (46%) patients in the HFpEF group and 387 (39%) patients in the no-HF group were obese (BMI $\geq$30 kg/m^2). Baseline characteristics of the respective cohorts are displayed in *Table 1* (stratified according to HFpEF or no-HF and the presence of obesity) and online supplementary *Table S2* (stratified according to HFpEF or no-HF only). HFpEF patients displayed the expected profile characterized by higher age, higher BMI, higher estimated plasma volume and a higher frequency of comorbidities like arterial hypertension, diabetes or atrial fibrillation compared to the no-HF group. Echocardiographically, HFpEF patients had higher mean E/E′ ratios, more pronounced left ventricular hypertrophy and more progressive LA dilatation. Markers of inflammation [CRP and interleukin (IL)-6] were also markedly higher in HFpEF patients.

In comparison to obese no-HF patients, obese HFpEF patients were older and had a higher prevalence of atrial fibrillation. As in the overall cohort, CRP and IL-6 tended to be higher in obese HFpEF patients, even when compared to obese no-HF patients. Lean HFpEF patients exhibited a comparable profile with higher markers of inflammation when compared to lean no-HF controls.

Despite being at comparable age and extent of comorbidities (except for the presence of diabetes), obese HFpEF patients had higher indices of cardiac mass, more progressive LA dilatation and higher E/E′ values as compared to their lean HFpEF counterparts.

Analytical validation of the Olink assay

Overall, 92 cardiovascular and inflammatory biomarkers were measured as described above. IL-6 and NT-proBNP were measured within the initial recruitment phase of our cohort and showed a good linear correlation to biomarker levels measured with the Olink system with IL-6 and NT-proBNP ($r = 0.813$, $P < 0.001$ and $r = 0.830$, $P < 0.001$, respectively) (online supplementary *Figure S1*). As NT-proBNP was part of the stratification of patients into HFpEF or no-HF, we removed brain natriuretic peptide from further analysis to avoid selection bias.

Protein levels in HFpEF patients with obesity

Out of the 91 analysed proteins, 44 proteins were significantly different in HFpEF patients, with 5 proteins being lower and 39 proteins being higher as compared to no-HF patients (online supplementary *Table S3*). Of those, nine proteins [adrenomedullin (ADM), tumour necrosis factor receptor superfamily member 10A (TNFRSF10A), tumour necrosis factor receptor superfamily member 11A (TNFRSF11A), tumour necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2), galectin-9 (Gal-9), spondin-2 (SPON2), kidney injury molecule (KIM1), thrombospondin-2 (THBS2), T-cell surface glycoprotein CD4 (CD4)] were significantly different between obese HFpEF patients as compared to both lean HFpEF or obese no-HF patients (online supplementary *Table S4*).

After adjustment for covariates, only ADM, CD4, Gal-9, THBS2 and TRAIL-R2 remained associated with HFpEF ($P < 0.001$, $P = 0.013$, $P = 0.008$, $P < 0.001$ and $P < 0.001$, respectively) and obesity ($P < 0.001$, $P = 0.005$, $P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively), with no significant interaction between HFpEF and obesity (*Figure 2*, online supplementary *Table S5*). Except for the association of CD4 and obesity, this remained true even when only patients with manifest HFpEF were included in the analysis (online

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Table 1 Baseline characteristics of patients with heart failure and preserved ejection fraction vs. patients without heart failure

Baseline characteristics	No heart failure (n = 999)		HFpEF (n = 999)		*P*-value* 1 vs. 2	*P*-value* 3 vs. 4	*P*-value* 1 vs. 3	*P*-value* 2 vs. 4
	Lean (n = 612)[1]	Obese (n = 387)[2]	Lean (n = 535)[3]	Obese (n = 464)[4]				
Age, years	64 (57, 70)	63 (57, 70)	69 (59, 74)	67 (59, 73)	1.00	1.00	<0.001	<0.001
Female sex, n (%)	226 (37)	157 (41)	201 (38)	203 (44)	1.00	1.00	1.00	1.00
Body mass index, kg/m^2	26.6 (24.5, 28.4)	33.1 (32.4, 36.1)	27.0 (25.1, 28.6)	33.6 (31.8, 36.1)	<0.001	<0.001	0.46	1.00
HFA-PEFF score, points	2 (2, 3) n = 495	2 (3, 4) n = 330	5 (4, 6) n = 420	5 (4, 6) n = 391	<0.001	1.00	<0.001	<0.001
Comorbidities, n (%)								
Arterial hypertension	451 (74)	353 (91)	463 (87)	425 (92)	<0.001	0.44	<0.001	1.00
Diabetes mellitus	30 (5)	146 (38)	115 (22)	181 (39)	<0.001	<0.001	<0.001	1.00
Atrial fibrillation history	7 (1)	9 (2)	40 (8)	53 (11)	1.00	1.00	<0.001	<0.001
Ever smoker	328 (54)	230 (59)	316 (59)	245 (53)	1.00	1.00	1.00	1.00
History of PCI	12 (2)	12 (3)	46 (9)	28 (6)	1.00	1.00	<0.001	1.00
History of CABG	19 (3)	4 (1)	42 (8)	27 (6)	1.00	1.00	0.019	0.006
Coronary artery disease	358 (58)	232 (60)	382 (71)	310 (67)	1.00	1.00	0.001	0.71
Laboratory findings								
eGFR, mL/min/1.73 m^2	84 (74, 97)	85 (73, 96)	82 (70, 94)	80 (66, 94)	1.00	1.00	0.032	0.001
eGFR <60 mL/min/1.73 m^2, n (%)	19 (3)	26 (7)	73 (14)	75 (16)	0.43	1.00	<0.001	0.001
Urea, mmol/L	5.4 (4.5, 6.5)	4.8 (5.7, 6.6)	5.6 (4.6, 6.8)	6.0 (4.9, 7.6)	0.37	0.011	0.73	0.038
Haemoglobin, mmol/L	9.0 (8.4, 9.5)	9.0 (8.4, 9.4)	8.8 (8.1, 9.3)	8.7 (8.1, 9.2)	1.00	1.00	<0.001	<0.001
Haematocrit, %	42 (40, 44)	42 (40, 44)	42 (39, 44)	41 (39, 44)	1.00	1.00	0.001	0.002
Plasma volume, mL	2622 (2402, 2843)	3135 (2919, 3377)	2688 (2487, 2866)	3154 (2943, 3410)	<0.001	<0.001	0.012	1.00
Total serum protein, g/L	73 ± 5	73 ± 4	72 ± 5	72 ± 5	1.00	1.00	0.017	0.078
White blood cell count, Gpt/L	6.6 (5.5, 8.0)	6.8 (5.7, 8.1)	6.9 (5.7, 8.3)	7.2 (6.1, 8.5)	1.00	0.23	0.83	0.024
HbA1c, %	5.6 (5.3, 5.9)	5.9 (5.5, 6.5)	5.6 (5.4, 6.0)	6.0 (5.5, 6.6)	<0.001	<0.001	0.60	1.00
HbA1c in diabetics, %	6.1 (5.9, 6.6)	6.6 (6.1, 7.3)	6.3 (5.9, 7.0)	6.7 (6.2, 7.2)	0.19	0.027	1.00	1.00
NT-proBNP, pg/mL	77 (60, 98)	79 (60, 102)	286 (182, 602)	266 (175, 490)	1.00	1.00	<0.001	<0.001
C-reactive protein, mg/L	1.4 (0.8, 2.8)	2.7 (1.5, 4.9)	1.9 (1.0, 4.0)	3.2 (1.7, 6.0)	<0.001	<0.001	<0.001	0.15
Interleukin-6, pg/mL	1.8 (1.5, 3.3)	2.9 (1.6, 4.6)	2.7 (1.5, 5.2)	3.5 (2.1, 5.9)	<0.001	<0.001	<0.001	0.001
Echocardiographic findings								
LV ejection fraction, %	63 (59, 66)	62 (59, 66)	60 (56, 65)	61 (56, 65)	1.00	1.00	<0.001	0.34
LV end-diastolic volume index, mL/m^2	45 (37, 57)	51 (40, 65)	49 (39, 62)	51 (41, 66)	<0.001	0.60	0.015	1.00
LV end-diastolic mass index, g/m^2	106 (89, 128) n = 591	128 (106, 151) n = 369	126 (105, 149) n = 526	137 (117, 169) n = 451	<0.001	<0.001	<0.001	<0.001
LA anteroposterior diameter index, mm/m^2	21 (19, 24) n = 598	24 (22, 26) n = 375	23 (21, 25) n = 530	25 (22, 27) n = 454	<0.001	<0.001	<0.001	<0.001
MV E velocity max, cm/s	0.61 (0.53, 0.74) n = 588	0.65 (0.52, 0.77) n = 370	0.69 (0.56, 0.84) n = 515	0.73 (0.60, 0.93) n = 441	1.00	0.016	<0.001	<0.001
MV E′ septal, cm/s	0.06 (0.05, 0.08) n = 480	0.06 (0.05, 0.08) n = 325	0.06 (0.05, 0.07) n = 389	0.06 (0.05, 0.07) n = 369	1.00	1.00	<0.001	0.038
MV E′ lateral, cm/s	0.09 (0.07, 0.11) n = 479	0.09 (0.07, 0.11) n = 324	0.09 (0.07, 0.11) n = 391	0.09 (0.07, 0.11) n = 370	1.00	1.00	0.061	1.00
MV E/E′ mean, ratio	7.8 (6.4, 9.7) n = 479	8.3 (6.8, 10.2) n = 325	9.1 (7.3, 12.1) n = 389	10.1 (8.0, 13.0) n = 371	0.59	0.004	<0.001	<0.001
TAPSE, mm	21 (16, 23) n = 239	21 (19, 24) n = 157	20 (18, 23) n = 232	21 (19, 24) n = 177	1.00	0.79	1.00	1.00
RA-RV pressure gradient, mmHg	21 (16, 26) n = 318	22 (15, 26) n = 184	25 (20, 30) n = 335	26 (16, 32) n = 249	1.00	1.00	<0.001	0.002

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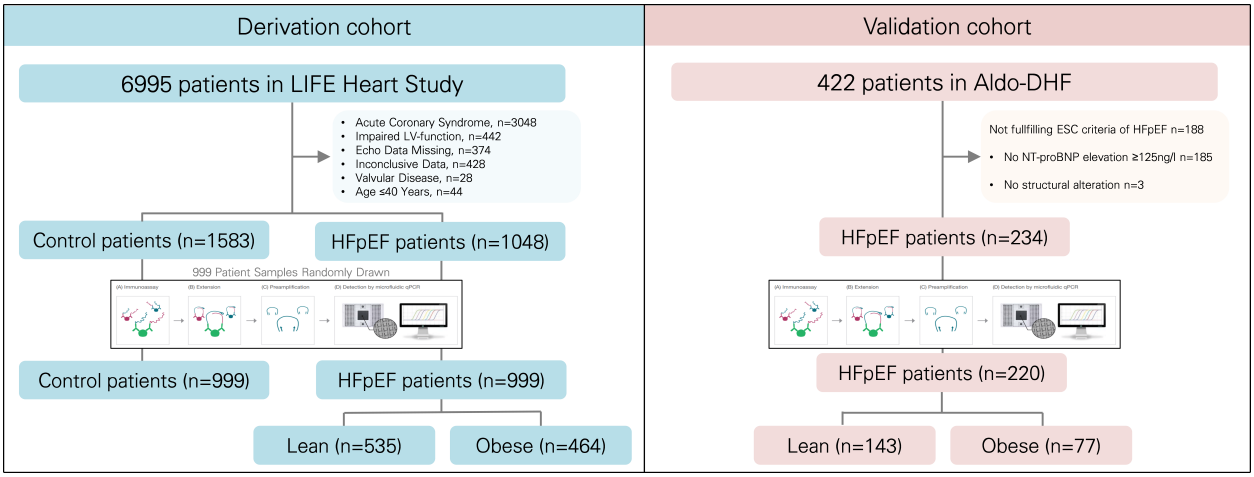

Figure 1 Study flow chart. ESC, European Society of Cardiology; HFpEF, heart failure with preserved ejection fraction; LV, left ventricular; NT-proBNP, N-terminal pro brain natriuretic peptide.

supplementary *Table S6*). Further, in a multivariable stepwise forward model including both baseline characteristics and the identified proteins from the Olink panel as independent covariates, all five proteins remained associated with the diagnosis of obese HFpEF in comparison to lean HFpEF (online supplementary *Table S7*).

Association of adrenomedullin, galectin-9, thrombospondin-2, CD4 and TRAIL-R2 with HFpEF severity and C-reactive protein

A protein score (primary component 1, PC1) derived from ADM, CD4, Gal-9, THBS2 and TRAIL-R2, as well as the individual proteins were all associated with higher HFA-PEFF scores, LA dilatation, left ventricular mass index and increased CRP (*Figure 3*), except for TRAIL-R2. Except for CD4, all other proteins showed an association with E/E′ even after adjustment for age, sex and renal function in the overall cohort. Furthermore, ADM showed the best linear correlation of all four identified proteins to estimated plasma volume in HFpEF patients ($r = 0.225$, $P < 0.001$). NT-proBNP did not show a correlation with estimated plasma volume ($P = 0.11$). Association of the proteins with known functional pathways as well as a network analysis based on partial correlation are displayed in online supplementary *Figures S2* and *S3*, respectively.

Association of HFpEF and obese HFpEF specific proteins with all-cause mortality in HFpEF patients

At a median follow-up of 7 years (IQR 5.2, 9.0), 113 HFpEF and 43 no-HF patients died (online supplementary *Figure S4*, log-rank $P < 0.001$). The association of the proteins with significantly different levels in HFpEF patients, with risk for all-cause death among patients with HFpEF, before and after adjustment for age, sex, renal function, smoking status, systolic blood pressure at admission, total cholesterol, presence of diabetes, CRP, NT-proBNP and the presence of coronary artery disease are displayed in *Table 2*.

In the subgroup of obese HFpEF patients of the five proteins uniquely higher expressed proteins ADM ($P = 0.013$) and CD4 ($P = 0.015$), as well as the PC1 ($P = 0.013$) were associated with all-cause mortality, following adjustment for age, sex, renal function, the presence of diabetes and the presence of coronary artery disease (*Graphical Abstract*). Interestingly, NT-proBNP (measured at timepoint of patient inclusion with a standard assay), which was prognostically relevant in the pooled cohort of HFpEF patients, was not able to predict survival in HFpEF patients with obesity ($P = 0.063$) (*Graphical Abstract*).

Validation of the identified proteins

Of the 422 patients included in the Aldo-DHF trial, 389 patients had measurements of the Olink Cardiovascular II panel available, and of those 220 had HFpEF according to the ESC criteria. Of those, 77 (35%) were obese. As outlined among other baseline characteristics in online supplementary *Table S8*, obese patients more frequently exhibited diabetes and had higher baseline glycated haemoglobin values. All five identified proteins which were higher expressed in obese HFpEF patients in the derivation cohort were also higher in obese HFpEF patients as compared to lean HFpEF patients in the validation cohort (online supplementary *Figure S5* and *Table S9*).

Discussion

In this large cohort study, we set out to identify circulating biomarkers that might be associated with a distinct obese phenotype of HFpEF. The main findings of the study are: (i) we identified and validated five proteins that were uniquely elevated in a clinically defined

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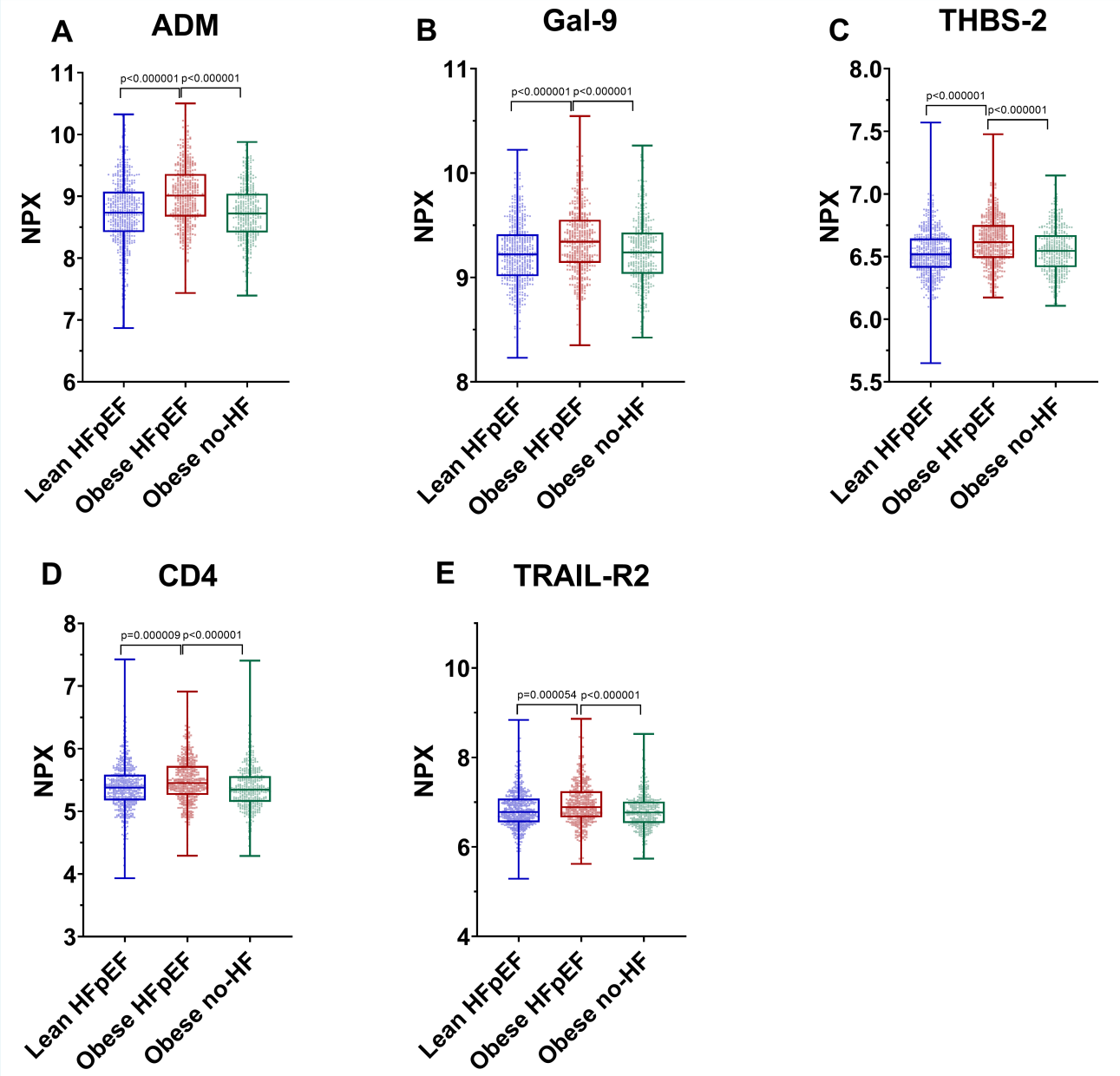

Figure 2 Differences in protein concentrations in adrenomedullin (ADM), galectin-9 (Gal-9), thrombospondin-2 (THBS2), T-cell surface glycoprotein CD4 and tumour necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) in obese heart failure with preserved ejection fraction (HFpEF) patients as compared to lean HFpEF and obese no-heart failure (HF) patients. NPX, normalized protein expression.

obese HFpEF phenotype, and (ii) these proteins reflect pathogenic factors in HF including volume expansion (ADM), fibrosis (THBS2) and inflammation (Gal-9, TRAIL-R2 and CD4), which might characterize an obese HFpEF phenotype, with (iii) some of those proteins being linked to mortality.

The theory of a distinct HFpEF phenotype linked to obesity has sparked large interest in recent years with reports associating it with a distinct haemodynamic profile[14] and the hypothesis that it might be associated with plasma volume expansion, accumulating fibrosis and enhanced inflammation when compared to other HFpEF phenotypes as proposed by Packer *et al.*[6,8,17] Our observations support this concept and expand it by linking the obesity HFpEF phenotype to a distinct biomarker signature. Importantly, in contrast to other studies that aimed to identify circulating protein markers of an obese HFpEF phenotype, we included a control cohort with obesity, but no evident HF to differentiate the pathognomonic effects of mere obesity from true obesity specific HFpEF features.[9,18] Further, we validated those findings in an independent prospective cohort of patients with HFpEF from a randomized trial.[15] In contrast to previous studies,

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Table 1 (Continued)

Baseline characteristics	No heart failure (n = 999)		HFpEF (n = 999)		P-value*	P-value*	P-value*	P-value*
	Lean (n = 612)[1]	Obese (n = 387)[2]	Lean (n = 535)[3]	Obese (n = 464)[4]	1 vs. 2	3 vs. 4	1 vs. 3	2 vs. 4
Medication, *n* (%)								
ACE-inhibitor/ARB	416 (68)	326 (84)	406 (76)	386 (83)	<0.001	0.18	0.12	1.00
Beta-blocker	350 (57)	243 (63)	386 (72)	371 (80)	1.00	0.18	<0.001	<0.001
Mineralocorticoid receptor antagonists	9 (2)	7 (2)	16 (3)	21 (5)	1.00	1.00	1.00	1.00
Diuretics	66 (11)	79 (20)	132 (25)	178 (38)	0.002	<0.001	<0.001	<0.001
Statins	273 (45)	164 (42)	276 (52)	240 (52)	1.00	1.00	0.79	0.27
Symptoms, *n* (%)								
Dyspnoea	140 (23)	150 (39)	161 (30)	203 (44)	<0.001	<0.001	0.22	1.00
NYHA class ≥III	23 (4)	31 (8)	29 (5)	61 (13)	<0.001	<0.001	0.91	1.00

ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; CABG, coronary artery bypass grafting; eGFR, estimated glomerular filtration rate (Modification of Diet in Renal Disease formula); HbA1c, glycated haemoglobin; HFpEF, heart failure with preserved ejection fraction; LA, left atrial; LV, left ventricular; MV, mitral valve; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; PCI, percutaneous coronary intervention; RA, right atrial; RV, right ventricular; TAPSE, tricuspid annular plane systolic excursion.
Superscripted numbers represent group abbreviations for statistical testing.
*P-values are adjusted for multiple testing (n = 41).

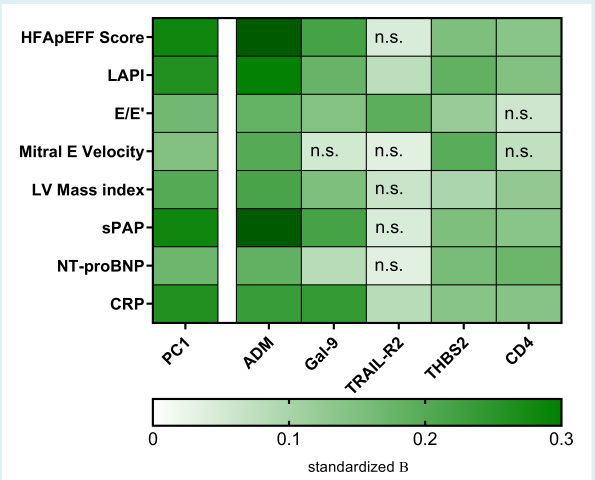

Figure 3 Associations of protein concentrations of adrenomedullin (ADM), galectin-9 (Gal-9), tumour necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2), thrombospondin-2 (THBS2) and T-cell surface glycoprotein CD4 with functional parameters. CRP, C-reactive protein; LAPI, left atrial anterior posterior index; LV, left ventricle; NT-proBNP, N-terminal pro brain natriuretic peptide; PC1, primary component 1; sPAP, systolic pulmonary artery pressure. All other results showed an adjusted *P*-value <0.05 after adjustment for age, sex and renal function.

we can now distinguish obesity-related alterations from primarily HFpEF-associated changes and identify volume expansion, fibrosis and inflammation as possible hallmarks of an obese HFpEF phenotype on a protein level.[9,19]

Adrenomedullin is a peptide hormone accredited with cardioprotective effects and leads to vasodilatation, inhibition of vascular structural remodelling, potentially reduced vascular permeability and natriuresis, which have been linked to impaired cardiovascular outcome.[20,21] Like BNP, ADM is also metabolized by neprilysin,[20] which is proposed to be overactivated in obese individuals. In contrast to BNP which mainly serves as a marker of intravascular congestion, ADM has been proposed as a marker of total fluid overload among HF patients incorporating intravascular as well as tissue congestion, which is possibly reflected in our cohort by the better correlation to total plasma volume than NT-proBNP among HFpEF patients. Circulating ADM levels are proposed as a counteracting response to volume overload and have been shown to decrease alongside successful decongestion in HF.[20,22] As observed in our cohort, ADM has been associated with echocardiographic parameters of diastolic impairment, likely mediated by increases in total volume.[23] Recently, increased levels of ADM were observed in patients with HFpEF when compared to healthy controls and a linear correlation of rising ADM levels with pulmonary capillary wedge pressures was described. Interestingly, as compared to controls, the HFpEF population investigated in this study was significantly heavier (BMI 34.1 vs. 27.5 kg/m^2, $P = 0.0003$) and might have potentially largely resembled a comparable obese HFpEF phenotype as analysed in our study.[24] Furthermore, ADM is expressed in

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Table 2 Prognostic association of different protein concentrations with outcome in patients with pre- and manifest heart failure with preserved ejection fraction

	Protein abbreviation	Univariable HR (95% CI)	Adjusted for covariates[a] HR (95% CI)
A disintegrin and metalloproteinase with thrombospondin motifs 13	ADAM-TS13	0.23 (0.11, 0.45)	0.55 (0.12, 2.40)
Adrenomedullin	ADM	3.10 (2.21, 4.36)	2.67 (1.71, 4.18)
Agouti-related protein	AGRP	2.23 (1.75, 2.84)	2.42 (1.74, 3.38)
Protein AMBP	AMBP	3.05 (1.30, 7.18)	2.54 (0.84, 7.65)
Angiopoietin-1	ANGPT1	0.23 (0.11, 0.45)	0.34 (0.16, 0.71)
Natriuretic peptides B	BNP	1.55 (1.36, 1.76)	1.37 (1.14, 1.65)
Carbonic anhydrase 5A, mitochondrial	CA5A	1.00 (0.85, 1.18)	0.98 (0.83, 1.16)
T-cell surface glycoprotein CD4	CD4	2.85 (1.82, 4.47)	2.66 (1.53, 4.60)
Cathepsin L1	CTSL1	3.39 (2.09, 5.48)	2.58 (1.46, 4.58)
Decorin	DCN	4.72 (2.92, 7.62)	2.14 (1.18, 3.90)
Fibroblast growth factor 21	FGF21	1.16 (1.02, 1.31)	1.14 (0.99, 1.31)
Fibroblast growth factor 23	FGF-23	1.34 (1.14, 1.57)	1.49 (1.16, 1.91)
Follistatin	FS	1.90 (1.28, 2.81)	1.09 (0.69, 1.72)
Galectin-9	Gal-9	3.68 (2.20, 6.16)	3.64 (1.93, 6.88)
Growth hormone	GH	1.08 (0.97, 1.19)	1.02 (0.92, 1.13)
Gastrotropin	GT	1.24 (0.98, 1.56)	1.15 (0.88, 1.51)
Osteoclast-associated immunoglobulin-like receptor	hOSCAR	3.51 (1.40, 8.83)	3.95 (1.43, 10.94)
Heat shock 27 kDa protein	HSP 27	1.66 (1.13, 2.43)	1.26 (0.82, 1.95)
Pro-interleukin-16	IL16	2.02 (1.37, 2.97)	1.76 (1.17, 2.66)
Interleukin-17D	IL-17D	1.59 (1.24, 2.05)	1.43 (0.95, 2.17)
Interleukin-1 receptor antagonist protein	IL-1ra	1.13 (0.83, 1.53)	1.28 (0.90, 1.83)
Interleukin-27	IL-27	4.28 (2.77, 6.63)	2.68 (1.65, 4.33)
Interleukin-4 receptor subunit alpha	IL-4RA	1.57 (1.24, 1.99)	1.54 (1.09, 2.18)
Interleukin-6	IL6	1.31 (1.15, 1.49)	1.24 (1.05, 1.47)
Kidney injury molecule 1	KIM1	1.51 (1.27, 1.78)	1.43 (1.16, 1.76)
Matrix metalloproteinase 12	MMP-12	1.80 (1.45, 2.22)	1.73 (1.34, 2.22)
Matrix metalloproteinase 7	MMP-7	2.37 (1.44, 3.92)	2.18 (1.22, 3.89)
Pappalysin-1	PAPPA	1.64 (1.31, 2.05)	1.37 (1.07, 1.75)
Proteinase-activated receptor 1	PAR-1	4.25 (2.57, 7.03)	4.13 (2.17, 7.84)
Platelet-derived growth factor subunit B	PDGF subunit B	0.22 (0.10, 0.49)	0.39 (0.16, 0.93)
Placental growth factor	PGF	2.36 (1.76, 3.15)	2.95 (1.61, 5.39)
Polymeric immunoglobulin receptor	PIgR	3.56 (1.11, 11.43)	2.29 (0.65, 8.08)
Prolargin	PRELP	6.93 (3.12, 15.38)	3.28 (1.29, 8.30)
Pentraxin-related protein PTX3	PTX3	1.50 (0.98, 2.28)	1.33 (0.82, 2.16)
Receptor for advanced glycosylation end products	RAGE	1.47 (0.97, 2.23)	1.21 (0.77, 1.90)
SLAM family member 7	SLAMF7	1.59 (1.22, 2.06)	1.34 (1.02, 1.77)
Spondin-2	SPON2	7.62 (2.24, 25.96)	5.03 (1.24, 20.52)
Proto-oncogene tyrosine-protein kinase Src	SRC	0.69 (0.51, 0.94)	0.88 (0.63, 1.23)
Thrombospondin-2	THBS2	3.57 (1.47, 8.67)	2.43 (0.94, 6.26)
Tumour necrosis factor receptor superfamily member 10A	TNFRSF10A	2.10 (1.44, 3.07)	2.36 (1.42, 3.92)
Tumour necrosis factor receptor superfamily member 11A	TNFRSF11A	1.75 (1.38, 2.22)	1.93 (1.31, 2.82)
Tumour necrosis factor receptor superfamily member 13B	TNFRSF13B	2.06 (1.41, 3.02)	1.85 (1.23, 2.77)
Tumour necrosis factor -related apoptosis-inducing ligand receptor 2	TRAIL-R2	1.64 (1.39, 1.93)	1.48 (1.20, 1.84)
Vascular endothelial growth factor D	VEGFD	1.06 (0.70, 1.60)	1.12 (0.78, 1.60)
Lymphotactin	XCL1	1.75 (1.32 2.34)	1.66 (1.12, 2.27)

Per NPX. CI, confidence interval; HR, hazard ratio.
[a]Adjusted for age, sex, renal function, smoking status, systolic blood pressure at admission, total cholesterol, presence of diabetes, C-reactive protein, N-terminal pro brain natriuretic peptide and presence of coronary artery disease.

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epicardial fat tissue[25] which has been proposed to show expansion and altered paracrine function especially among HFpEF patients with obesity[26] and might account for increased circulating ADM levels in our cohort.

Thrombospondin-2 is a matricellular protein and has been proposed to contribute to counteract cardiac fibrosis and matrix integrity during states of cardiac pressure overload.[27] THBS2 is also expressed in adipose tissue and might be up-regulated by nutritionally induced obesity.[28] It has been shown to have at least partial cardiac origin, association with the development of HF[29] and that it might be a marker of disease severity among patients with HFpEF.[27] Due to its role in matrix remodelling, it has been proposed as a possibly relevant mediator in the transition from mere cardiac fibrosis to eminent HF, explaining its overexpression in our cohort in which a large proportion of patients was marked by structural alterations in the absence of evident symptoms (pre-HFpEF).[30]

Galectin-9 is mainly expressed by endothelial cells, macrophages, and in particular T lymphocytes and induces apoptosis in subsets of differentiated T cells, particularly in Th1 and Th17 cell.[31] Neutralization of Gal-9 leads to enhanced inflammatory responses.[32,33] It has been associated with extracellular matrix remodelling and has repeatedly been associated with the prediction of incipient HF in patients at risk for cardiovascular disease.[19,34] In obese rodents, Gal-9 expression increases vastly in visceral adipose tissue and has been linked to an immunoregulatory effect.[35] Increasing levels of Gal-9, specifically observed in HFpEF patients with obesity, might be a regulatory effect to cope with overexpression of inflammation above and beyond the extent of obesity-related inflammation, as Gal-9 levels were significantly higher in HFpEF patients with obesity when compared to lean HFpEF or no-HF patients with obesity.

CD4 is a glycoprotein found on the surface of immune cells like T-helper cells and has been associated with progression of HF and increased fibrosis.[36] CD4 has been shown to infiltrate visceral adipose tissue and correlate with the extent of obesity. In obese patients, CD4 T-cells in visceral adipose tissue produce higher amounts of interferon-gamma as compared to lean controls, indicating an altered inflammatory response.[37] Deficiency of CD4 T-cells has been associated with reduced adipose tissue inflammation and enhanced insulin sensitivity, suggesting a role of CD4 in adipose tissue inflammation and transformation to metabolic syndrome associated with obesity,[37] pathways that have been proposed in the development and uphold of an obese HFpEF phenotype.[6,17,26] However, it is not clear whether adipose tissue inflammation is cause or causation of a systemic inflammatory response observed in HFpEF patients.[17] The uniquely higher expression of CD4 in our HFpEF population with obesity suggests effects that are above the levels that one would expect for mere obesity.

Heart failure with preserved ejection fraction has been associated with increased circulating biomarkers that link both fibrosis and inflammation (i.e. ST2, SPON-1)[38] and recent studies have shown that there is a specific subpopulation of HFpEF patients that is marked by an inflammatory cluster.[9,18] In this context, TRAIL-R2 has been a central protein among these inflammatory clusters.[9] Inflammatory clusters were characterized by highest BMI values but in the absence of an obese control group it was not possible to distinguish from obesity-mediated inflammation.[10] This is likely to be observed in any disease population when just stratified by BMI, and true inflammatory pathways that might only be associated with a specific obese HFpEF phenotype would need further confirmation. We were able to confirm the elevation of TRAIL-R2 and were also able to link TRAIL-R2 to estimates of increased LA pressure as described before among HFpEF patients.[9] The high expression of inflammatory proteins in our cohort (CD4, Gal-9 and TRAIL-R2) is in line with previous reports that have constantly highlighted the important role of systemic inflammation in increased myocardial collagen deposition, resulting in decreased ventricular compliance.[39] Alterations in TRAIL-R2, CD4 and ADM have been described in HFpEF patients before,[9] suggesting an association between systemic inflammation and volume expansion,[40] with patients that show highest values in those three proteins likely sharing the worst prognosis. In fact, the crucial role of inflammation and volume expansion especially among HFpEF patients with obesity is underlined by the fact that out of the five proteins uniquely higher in these patients, only proteins linked to inflammation (CD4) and volume expansion (ADM) were associated with impaired outcome. This provides a potential rationale for the therapy of these patients with sodium–glucose co-transporter 2 inhibitors, as they might attenuate visceral fat and systemic inflammation, limit cardiac fibrosis and reduce renal tubular sodium reabsorption, three potential hallmarks of obese HFpEF.[41]

Given the nature of proximity extension assays, the current study cannot provide generally applicable cut-off values for identified biomarkers for clinical routine.[16] The observed linear association with disease severity and outcomes allows for some interpretation of biomarker levels even in absence of established cut-off values. However, the role of those biomarkers when measured in clinical routine remains to be determined. Further, most of the identified markers show at least some association to pericardial fat, which is increased in HFpEF patients,[42] investigating this association might further facilitate the understanding of the obese HFpEF phenotype.

Strengths and limitations

Strengths of our study include the prospective, large cohort which is comprehensively phenotyped and has a long-term follow-up supported by a validation cohort from a randomized controlled trial. In contrast to many other cohorts comparing HFpEF patients with healthy controls, our control cohort of no-HF patients consisted of patients with elevated cardiovascular risk and an abundance of relevant comorbidities, that however did not lead to the expression of a HFpEF phenotype in this cohort. We were not only able to identify proteins associated with HFpEF but also with an obese HFpEF phenotype and link these findings to disease severity as well as outcomes. However, we did not include a cohort of patients with HF with reduced ejection fraction and we cannot exclude that alterations in the protein profile might be attributable to obese HF in general rather than HFpEF in particular. Further, the analysis was

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limited to 91 proteins and larger mass spectrometry-based proteomics might provide even further insights in the obese HFpEF phenotype in future research.

In contrast to other studies, we also included pre-stage HFpEF patients which might explain the relatively favourable outcome, as compared to other studies that included HFpEF patients based on previous HF hospitalizations, likely at a later stage of disease. We were able to validate our findings of differences in protein expression in HFpEF patients according to state of obesity in a contemporary prospective cohort from a randomized clinical trial focusing on HFpEF patients. However, the number of patients with obesity-associated HFpEF was limited in the validation cohort ($n = 77$) and our data are to be considered as hypothesis generating.

Conclusion

In a large set of patients with HFpEF and no-HF we identified proteins uniquely associated with an obese HFpEF phenotype and validated these findings in an independent cohort. Those proteins resemble the potentially relevant pathways of plasma volume expansion (ADM), fibrosis (THBS2) and inflammation (Gal-9, CD4 and TRAIL-R2) and were linked to disease progression. Those identified markers nourish our understanding of an obese HFpEF phenotype and might lead to new insights in terms of prognostication as well as enhance our pathophysiological understanding.

Supplementary Information

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

Acknowledgment

Open access funding enabled and organized by Projekt DEAL.

Funding

This study was funded by a cooperative grant from HELIOS and the University of Leipzig. LIFE-Heart was funded by the European Union, the European Regional Development Fund (ERDF) and by funds of the Free State of Saxony within the framework of the excellence initiative.

The Aldo-DHF study was supported by the German Competence Network of Heart Failure. Aldo-DHF was funded by the Federal Ministry of Education and Research Grant 01GI0205 [clinical trial program Aldo-DHF (FKZ 01KG0506)]. The University of Göttingen was the formal sponsor.

Conflict of interest: R.W. received honoraria as a consultant or speaker from AstraZeneca, Bayer, BMS, Boehringer Ingelheim, CVRx, Daiichi, Medtronic, Novartis, Pfizer, Pharmacosmos and Servier. M.S. receives funding from Pfizer Inc. for a project not related to this research. F.E. received honoraria as a consultant or speaker from AstraZeneca, Bayer, Merck, MSD, Boehringer Ingelheim, Novartis, Pfizer, Pharmacosmos, Vifor Pharma and Servier. M.B. received honoraria as a consultant and speaker from Amgen, AstraZeneca, Bayer, Boehringer Ingelheim, Lilly, Novo Nordisk, Novartis, and Sanofi. P.L. is a consultant to ReCor Medical and Medtronic. All other authors have nothing to disclose.

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