ESC HEART FAILURE
ESC Heart Failure 2022; **9**: 3961–3972
Published online 18 August 2022 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/ehf2.14116

ORIGINAL ARTICLE

Nitric oxide metabolites: associations with cardiovascular biomarkers and clinical parameters in patients with HFpEF

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Abstract

Aims Heart failure with preserved ejection fraction (HFpEF) is one of the most rapidly growing cardiovascular health burden worldwide, but there is still a lack of understanding about the HFpEF pathophysiology. The nitric oxide (NO) signalling pathway has been identified as a potential key element. The aim of our study was to investigate markers of NO metabolism [L-arginine (L-Arg), homoarginine (hArg), and asymmetric and symmetric dimethylarginine (ADMA and SDMA)], additional biomarkers [N-terminal pro-B-type natriuretic peptide (NT-proBNP), endothelin-1 (ET-1), mid-regional pro-adrenomedullin (MR-proADM), copeptin, and high-sensitivity C-reactive protein (hsCRP)], and the endothelial function in an integrated approach focusing on associations with clinical characteristics in patients with HFpEF.

Methods and results Seventy-three patients, prospectively enrolled in the 'German HFpEF Registry', were analysed. Inclusion criteria were left ventricular ejection fraction (LVEF) $\geq$ 50%; New York Heart Association functional class $\geq$ II; elevated levels of NT-proBNP $>$ 125 pg/mL; and at least one additional criterion for structural heart disease or diastolic dysfunction. All patients underwent transthoracic echocardiography, cardiopulmonary exercise testing, and pulse amplitude tonometry (EndoPAT™). Patients were categorized in two groups based on their retrospectively calculated HFA-PEFF score. Serum concentrations of L-Arg, hArg, ADMA, SDMA, NT-proBNP, ET-1, MR-proADM, copeptin, and hsCRP were determined. Patients had a median age of 74 years, 47% were female, and median LVEF was 57%. Fifty-two patients (71%) had an HFA-PEFF score $\geq$ 5 (definitive HFpEF), and 21 patients (29%) a score of 3 to 4 (risk for HFpEF). Overall biomarker concentrations were 126 ± 32 μmol/L for L-Arg, 1.67 ± 0.55 μmol/L for hArg, 0.74 (0.60;0.85) μmol/L for SDMA, and 0.61 ± 0.10 μmol/L for ADMA. The median reactive hyperaemia index (RHI) was 1.55 (1.38;1.87). SDMA correlated with NT-proBNP (r = 0.291; P = 0.013), ET-1 (r = 0.233; P = 0.047), and copeptin (r = 0.381; P = 0.001). ADMA correlated with ET-1 (r = 0.250; P = 0.033) and hsCRP (r = 0.303; P = 0.009). SDMA was associated with the left atrial volume index (β = 0.332; P = 0.004), also after adjustment for age, sex, and comorbidities. Biomarkers were non-associated with the RHI. A principal component analysis revealed two contrary clusters of biomarkers.

Conclusions Our findings suggest an impaired NO metabolism as one possible key pathogenic determinant in at least a subgroup of patients with HFpEF. We argue for further evaluation of NO-based therapies. Upcoming studies should clarify whether subgroups of HFpEF patients can take more benefit from therapies that are targeting NO metabolism and pathway.

Keywords HFpEF; Nitric oxide; Arginine; SDMA; Endothelial dysfunction

Received: 4 April 2022; Revised: 6 July 2022; Accepted: 4 August 2022
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Introduction

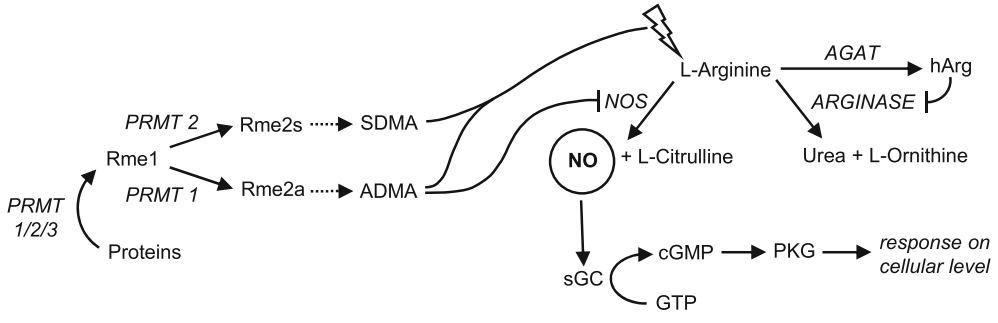
Heart failure with preserved ejection fraction (HFpEF) accounts for half of all heart failure (HF) cases, but there is still a lack of understanding about its pathophysiology.[1,2] The concept of increased left ventricular (LV) afterload as primary cause of HFpEF has been abandoned. Instead, the clinical syndrome is discussed to be the consequence of a comorbidity-driven systemic proinflammatory state that leads to microvascular endothelial inflammation and a reduced bioavailability of nitric oxide (NO).[3] With a lower NO bioavailability, the NO–soluble guanylate cyclase (sGC)–cyclic guanosine monophosphate (cGMP)–protein kinase G (PKG) axis is suppressed. The lower PKG activity causes the development of cardiac hypertrophy and increased cardiomyocyte resting tension due to a hypophosphorylation of titin.[3]

Nitric oxide is a signalling molecule synthesized from the amino acid L-arginine (L-Arg) by nitric oxide synthases (NOS).[4] Besides L-Arg, other arginine derivatives are involved in NO metabolism (see *Figure 1*). Methylarginines are formed within post-translational modifications of protein-bound arginine residues by protein arginine N-methyltransferases. Asymmetric dimethylarginine (ADMA) acts as direct inhibitor of the NOS and can therefore lower the NO bioavailability. Symmetric dimethylarginine (SDMA) has no direct influence on the NOS but can cause a reduced L-Arg uptake through competitive transport via the cationic amino acid transporter system. Thus, the substrate availability for the formation of NO from L-Arg decreases.[5] Homoarginine (hArg) is a metabolite formed from L-Arg and lysine by the L-arginine:glycine amidinotransferase (AGAT) and may act as alternative NOS substrate. Moreover, hArg can inhibit the enzyme arginase and thereby augment L-Arg pools.[6] Various preclinical studies have shown that oxidative stress and a reduced NO production were associated with an impaired ventricular relaxation and the development of diastolic dysfunction.[7,8] The reduced NO production was largely triggered by a dysfunctional NOS.[7] It was demonstrated that systemic and cardiovascular features of HFpEF could be induced by inhibiting the constitutive NOS.[8]

Besides its role in regulating myocardial contractility and relaxation, NO is essential for endothelial function including the regulation of vascular tone, haemostasis, and immunity, whereas endothelial dysfunction (ED) is a pathological state characterized by NO impairment, inflammation, and atherosclerosis.[9] Several studies could demonstrate ED in patients with HFpEF.[10,11] The endothelial function may be assessed through invasive or non-invasive methods. Infusion of vasoactive substances like acetylcholine combined with coronary angiography was one of the first methods. However, due to its invasiveness and associated risks, this method is not suitable for screening larger populations. Consequently, non-invasive methods like flow-mediated vasodilation (FMD) or peripheral arterial tonometry (PAT) were developed. PAT is a relatively new method based on plethysmographic recordings of changes in the peripheral arterial tone caused by an induced reactive hyperaemia.[9]

The use of L-Arg/NO metabolites like L-Arg, hArg, ADMA, or SDMA as biomarkers in the cardiovascular system has increasingly being investigated.[12] Whereas L-Arg and hArg were described as protective markers, ADMA and SDMA are seen as risk markers that impair NO metabolism.[12] In previous studies, L-Arg was no independent biomarker for cardiovascular events or total mortality, whereas elevated ADMA and SDMA concentrations independently predicted total and cardiovascular mortality.[12–14] A low plasma hArg concentration

Figure 1 Synthesis and interactions of methylated arginines, nitric oxide, and nitric oxide signalling pathway. Symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA) are formed within post-translational modifications of protein-bound arginine residues by protein arginine N-methyltransferases (PRMT). ADMA is a direct inhibitor of nitric oxide synthases (NOS). NOS synthesize nitric oxide (NO) from L-arginine. ADMA and SDMA can cause a reduced L-arginine uptake through a competitive transport via the cationic amino acid transporter system and thereby reduce substrate availability for NO synthesis. Homoarginine (hArg) is formed from L-arginine and lysine by the L-arginine:glycine amidinotransferase (AGAT). Homoarginine can inhibit the enzyme arginase and thereby augment L-arginine pools. A lower NO bioavailability leads to a suppression of the NO–soluble guanylate cyclase (sGC)–cyclic guanosine monophosphate (cGMP)–protein kinase G (PKG) axis. Rme1, monomethylated arginine residues; Rme2a, asymmetric dimethylated arginine residues; Rme2s, symmetric dimethylated arginine residues.



was identified as independent predictor of mortality, too.[15] With regard to the concept of a reduced NO bioavailability as a possible mechanism responsible for the development of HFpEF, NO metabolism and pathway gained interest as potential therapeutic targets in patients with HFpEF. To date, clinical trials that investigated the effects of inorganic nitrites (INDIE-HFpEF trial, 2018), organic nitrates (NEAT-HFpEF trial, 2015), and sGC stimulators (VITALITY-HFpEF trial, 2020) failed to improve the outcome of HFpEF patients.[16–18] Moreover, although the effects of methylarginines on NO synthesis have been widely investigated, there is still an overall lack of specific therapeutic strategies against elevated ADMA and SDMA concentrations.[19] Besides direct targeting of methylarginines, treatment of their adverse effects is proposed as another approach.[14] L-Arg has been widely supposed as 'antidote' of ADMA.[14] Although the administration of L-Arg and citrulline improved right ventricular function in patients with HFpEF, studies investigating the effect of L-Arg supplementation on overall cardiovascular outcome remain to be conducted.[14,20]

The natriuretic peptides B-type natriuretic peptide (BNP) and N-terminal pro-B-type natriuretic peptide (NT-proBNP) are established biomarkers in HF diagnostics.[2] However, their single use in HFpEF diagnostics is being discussed, as BNP and NT-proBNP are often affected by comorbidities.[2] Besides natriuretic peptides, there are other biomarkers like endothelin-1 (ET-1), mid-regional pro-adrenomedullin (MR-proADM), copeptin, and high-sensitivity C-reactive protein (hsCRP), whose role in patients with HFpEF is increasingly being investigated.[21–24] ET-1 is the overall predominant and most relevant isoform from the endothelin family and has highly potent vasoconstrictorical effects. ET-1 is synthesized and released mainly through the vascular endothelium.[25] Hypoxia, ischaemia, or an increased shear stress may stimulate the synthesis. ET-1 is also released by cardiomyocytes and is supposed to induce profibrotic remodelling processes in the myocardium.[26] A prognostic benefit of ET-1 was recently described in patients with HFpEF.[21] Arginine-vasopressin (AVP), also known as antidiuretic hormone (ADH), is a central hormone in controlling osmotic homeostasis. States of hypovolaemia and hypotension are known as main stimuli for an ADH secretion from the neurohypophysis. In states of stress, for example, in acute myocardial infarction, the secretion may be triggered.[27] ADH induces the reabsorption of water and a strong vasoconstriction. Due to its small size and instability, the more stable copeptin as the C-terminal part of the pre-pro-vasopressin is determined in laboratory analysis.[27] Adrenomedullin (ADM) is a vasodilatative peptide originally discovered in the adrenal medulla. Today, it is known that ADM is secreted by almost all body tissues including endothelial cells.[28] ADM is rather unstable. Thus, the mid-regional pro-adrenomedullin (MR-proADM) is determined in immunoassays, which occurs in equivalent concentrations.[27] Studies that investigated MR-proADM in patients with HFpEF showed only small or no diagnostic or prognostic benefits.[23,24] CRP is an acute-phase protein typically released by hepatocytes during inflammatory processes or when tissue is damaged. CRP seems to have both diagnostic and prognostic benefits in HFpEF patients.[22]

The aim of our study was to investigate markers of NO metabolism (including L-Arg, hArg, ADMA, and SDMA), additional cardiovascular biomarkers (including NT-proBNP, ET-1, copeptin, MR-proADM, and hsCRP), and the endothelial function in an integrated approach focusing on associations with clinical characteristics in patients with HFpEF.

Methods

Study population

Between August 2016 and June 2020, patients with HFpEF were prospectively enrolled in the 'German HFpEF Registry'. Inclusion criteria were defined in accordance with the 2016 ESC HF Guidelines: (i) left ventricular ejection fraction (LVEF) $\geq$ 50%; (ii) age $\geq$ 18 years; (iii) New York Heart Association (NYHA) functional class $\geq$ II; (iv) elevated levels of NT-proBNP $>$ 125 pg/mL; and (v) at least one additional criterion for structural heart disease or diastolic dysfunction [left ventricular mass index (LVMI) $\geq$ 115 g/m^2 for men and $\geq$95 g/m^2 for women; left atrial volume index (LAVI) $>$ 34 mL/m^2; left ventricular filling index (E/e' mean) $\geq$ 13; and early diastolic mitral annular velocity (e' mean) $<$ 9 cm/s]. It should be noted that the initial LVEF inclusion criterion of the registry was an LVEF $\geq$ 45%. To meet ESC criteria, only patients with an LVEF $\geq$ 50% were considered in this analysis. Exclusion criteria were (i) acute coronary syndrome during the past 3 months; (ii) cardiac surgery/percutaneous intervention during the past 3 months; and (iii) haemodynamic relevant pericardial disease. Patients were predominantly recruited at an outpatient setting at the Charité University Hospital, Department of Internal Medicine and Cardiology, Campus Virchow Klinikum, Berlin, Germany. For the present study, patients were excluded if they could not perform a maximal exercise testing, indicated by a maximum respiratory exchange rate (RER) $<$ 1 in cardiopulmonary exercise testing (CPET). The study complies with the Declaration of Helsinki. The Ethics Committee of Charité University Hospital approved the research project, and written informed consent was obtained from all subjects.

Clinical characteristics

The following data were collected from all patients: demographics, cardiovascular risk factors and comorbidities, body mass index (BMI), waist and hip size, blood pressure at rest,

NYHA functional class, and medications. Patients with a BMI $\geq$ 30 kg/m^2 were considered as obese. Anaemia was defined as haemoglobin (hb) levels $<$ 12 g/dL in women and $<$13 g/dL in men. HF signs and symptoms were assessed.

Blood sampling and laboratory analysis

Blood samples were taken from the patients in a sitting or lying position after a resting period of at least 5 min. The samples were then transferred to the study centre's laboratory and centrifuged for 10 min at 18°C and 600 *g*. The supernatant was pipetted off and stored at −80°C until further analysis. Some laboratory parameters were directly analysed from the taken samples, including hb and creatinine. Estimated glomerular filtration rate (eGFR) was determined using the CKD-EPI Creatinine Equation. L-Arg, hArg, ADMA, and SDMA were quantified from the stored serum samples using validated liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS). In brief, 25 μL of serum were diluted with stable isotope-labelled internal standards of the analytes solved in 100 μL methanol. After protein precipitation and evaporation of supernatants to dryness, analytes were converted to their butyl ester derivatives and subjected to positive electrospray ionization (ESI+) LC–MS/MS. Analyte concentrations were calculated from calibration curves for four concentration levels of each analyte, and quality controls (QCs) were run in two concentration levels in triplicates. All coefficients of variation and bias were $\leq$10% for QC (low) and $<$4% for QC (high).[29,30] ET-1 levels were measured by solid-phase sandwich ELISA (R&D Systems, Minneapolis, USA). MR-proADM levels were also analysed by sandwich ELISA (Elabscience Biotechnology, Wuhan, China). NT-proBNP was measured using an electro-chemiluminescence immunoassay (ECLIA), and hsCRP was determined by a turbidimetric immunoassay method.

Echocardiography

All study participants underwent transthoracic echocardiography at rest using Philips EPIQ 7 ultrasound system (Philips Medical Systems, Andover, Massachusetts, USA). In parasternal long axis, left ventricular end-diastolic diameter (LVEDD), end-diastolic interventricular septal thickness (IVSED), and end-diastolic posterior wall thickness (PWED) were determined. To assess the LVMI, the mass of the left ventricle (LV) was calculated from the collected LVEDD, IVSED, and PWED and then related to the body surface area (BSA). Additionally, relative wall thickness (RWT) was calculated from LVEDD and PWED. The left atrial end-systolic volume (LAESV) was determined using biplane method. Relating LAESV to BSA, LAVI was received. The end-diastolic and end-systolic volumes (EDV and ESV) of the LV were determined biplane using Simpson's disk summation method. Subsequently, LVEF was calculated from EDV and ESV. E wave and A wave were measured in pulsed wave Doppler to evaluate the LV inflow profile. Lateral and septal early diastolic mitral annulus velocities (e' lateral and e' septal) were determined in tissue Doppler, and the mean velocity (e' mean) was derived. In addition, the LV filling index (E/e' mean) was calculated from the collected parameters.

Calculating the HFA-PEFF score

In 2019, the Heart Failure Working Group (HFA) of the ESC presented a new HFA-PEFF diagnostic algorithm with HFA-PEFF score to simplify and structure HFpEF diagnostics.[31] We calculated the HFA-PEFF score for every patient based on available data (data on peak velocity of the tricuspid regurgitation and global longitudinal strain were non-available), as previously described.[31] In brief, 0–2 points were assigned in three domains (biomarker, functional, and morphological) and a total score was derived (0–6 points). An HFA-PEFF score $\geq$ 5 implies 'definitive HFpEF', a score of 2–4 points reflects 'risk for HFpEF' with recommendation for further diagnostics, and an HFA-PEFF score $\leq$ 1 makes the presence of HFpEF unlikely.[31]

Assessing endothelial function

To assess the patients' endothelial function, the EndoPAT™ 2000 system (Itamar Medical Ltd., Caesarea, Israel) was used. Finger sensors were attached to the right and left index fingers in accordance with the device manufacturer's specifications.[32] The current blood pressure was determined on the non-dominant arm. The non-dominant arm was chosen as measurement arm; the dominant arm served as internal control. Patients were advised to relax during the measurement. Measurement was started by an initial recording of a resting PAT signal for 5 min. Thereafter, the occlusion on the non-dominant measurement arm was induced by inflating the occlusion cuff approximately 50 mmHg above the previously determined systolic blood pressure. The occlusion was maintained for 5 min and then completely released. The following reactive hyperaemia signal was recorded for another 5 min. Finally, the EndoPAT™ software calculated the reactive hyperaemia index (RHI). The RHI results from the PAT signal post- to pre-occlusion in the occluded arm to the PAT signal post- to pre-occlusion in the control arm, multiplied with a baseline correction factor.[32] Based on a validation study, RHI cut-off values were defined. Accordingly, an RHI $>$ 1.67 indicates normal endothelial function and an RHI $\leq$ 1.67 ED. The RHI may also be given in its natural logarithm (lnRHI). Corresponding cut-off values are lnRHI $>$ 0.51 for normal endothelial function and lnRHI $\leq$ 0.51 for ED.[32]

Cardiopulmonary exercise testing and 6 min walk test

Exercise intolerance is one of the cardinal manifestations of HF. CPET is a non-invasive, integrative test to define the maximum exercise capacity by measurement of peak oxygen uptake (peakVO_2). A peakVO_2 of 14 mL/kg/min could be validated as prognostic cut-off in patients with HFpEF.[33,34] In our study, CPET was performed on a cycle ergometer (ergoselect 100, ergoline, Bitz, Germany) under the use of the MetaLyzer® 3B-R3 spiroergometry system and MetaSoft® Studio software (CORTEX Biophysik GmbH, Leipzig, Germany). The test followed a predefined protocol (resting phase, starting workload 20 W, stepwise 10 W increment every minute). Respiratory flow parameters were recorded as breath-by-breath data, including oxygen consumption (VO_2), carbon dioxide production (VCO_2), and minute ventilation (VE). A 12-channel electrocardiogram was recorded continuously, and blood pressure was measured at rest and every 2 min. The patients were encouraged to exercise to maximum exhaustion. The exercise was terminated in accordance with defined absolute and relative termination criteria. Patients were asked about their subjective degree of exhaustion at the time the exercise was discontinued (Borg scale 6–20). PeakVO_2 was determined as the highest VO_2 value within the last 30 s of exercise (average of breath-by-breath values). Maximum RER and exercise ventilatory efficiency (VE/VCO_2 Slope) were calculated. A standardized 6 min walk test (6MWT) was performed. Patients were asked about their subjective degree of exhaustion at the end of the test (Borg scale 6–20).

Statistical analysis

Based on the achieved HFA-PEFF scores, patients were categorized in two groups (definitive HFpEF and risk for HFpEF). To determine if normal distribution was present, the sample was checked visually by using histograms, boxplots, and qq-plots. Moreover, the difference between mean and median as well as skewness were rated. Continuous, normally distributed variables were expressed as mean ± standard deviation, and non-normally distributed variables as median and quartiles (first quartile; third quartile). Categorical variables were reported as absolute values and percentages. For comparison between groups, Pearson's χ^2 test was used for categorical variables and *t*-test (with normal distribution) or Mann–Whitney *U* test (no normal distribution) for continuous variables. Spearman's correlation coefficient was used to evaluate relationships between the markers. To assess possible associations between the markers and parameters of echocardiography, exercise capacity, and endothelial function, a simple linear regression analysis was performed (Model 1). Regression coefficients were shown in a heatmap. For significant associations found in Model 1, a multiple linear regression was performed. In Model 2, the variables age and sex were added. Model 3 was additionally adjusted by the variables BMI, atrial fibrillation (AFib), eGFR, and hb. A principal component analysis (PCA) was performed to assess principal components from the biomarkers and the RHI. All analyses were performed using the software IBM SPSS Statistics 25.0 (Armonk, NY, USA), and a P-value < 0.05 was considered statistically significant.

Results

Population characteristics

Of 131 patients included in the registry, 121 had an LVEF $\geq$ 50%. Complete data of medical history, physical examination, echocardiography, CPET, and EndoPAT™ were available in 98 patients. In seven patients, EndoPAT™ results were of poor quality. Thirteen patients reached a maximum RER < 1; from five patients were no stored serum samples available. Thus, a total of 73 patients could be included. Fifty-two patients (71%) had a retrospectively calculated HFA-PEFF score $\geq$ 5 (definitive HFpEF), and 21 patients (29%) a score of 3 to 4 (risk for HFpEF). As shown in *Table 1*, the median age of the study population was 74 years and 47% were female. Overall LVEF was 57 (55;60) %, LAVI 42 (34;53) mL/m^2, LVMI 112 (85;127) g/m^2, e' mean 6.7 ± 1.7 cm/s, and E/e' mean 12.2 (10.2;16.6). Fifty-eight patients (79.5%) were in NYHA class II, and 15 patients (20.5%) in NYHA class III. All patients were medicated according to standard of care in 2016–20.[2] Patients with definitive HFpEF reported a lower sleep duration [6 (6;7) vs. 7 (6.3;8) h; P = 0.030]. Moreover, patients with definitive HFpEF had an ischaemic cerebral event in their medical history more frequently than patients at risk for HFpEF (30.8% vs. 4.8%; P = 0.017). The eGFR in patients with definitive HFpEF tended to be lower (64.2 ± 16.6 vs. 72.9 ± 22.1 mL/min/1.73 m^2; P = 0.070). However, the eGFR difference was not statistically significant. Overall, patients showed typical HF signs and symptoms. As the only echocardiographic parameter, LAVI was significantly higher in patients with definitive HFpEF [47 (38;58) mL/m^2] compared with patients at risk for HFpEF [33 (26;38) mL/m^2; $P < 0.001$]. Overall peakVO_2 was 15.3 (12.9;18.8) mL/kg/min, and 6MWT walk distance was 440 ± 100 m.

Biomarker concentrations and reactive hyperaemia index

Biomarker concentrations are shown in *Table 2*. Overall concentrations were 126 ± 32 μmol/L for L-Arg,

Table 1 Baseline characteristics

	All patients (n = 73)	HFpEF (n = 52)	Risk for HFpEF (n = 21)	P-value
HFA-PEFF score	5 (4;6)	6 (5;6)	4 (4;4)	<0.001
Age (years)	74 (69;78)	76 (69;78)	72 (65;76)	0.059
Female sex, n (%)	34 (46.6)	25 (48.1)	9 (42.9)	0.797
Body mass index (kg/m^2)	27.6 (24.3;32.1)	27.9 (24.3;32.6)	26.8 (24.3;29.4)	0.414
Waist size (cm)	101 ± 16	102 ± 15	100 ± 18	0.742
Hip size (cm)	105 (98;112)	106 (97;113)	102 (99;111)	0.779
Systolic blood pressure (mmHg)	137 ± 19	135 ± 17	143 ± 22	0.107
Sleep duration (h)	6.5 (6.0;7.3)	6.0 (6.0;7.0)	7.0 (6.3;8.0)	0.030
RHI	1.55 (1.38;1.87)	1.52 (1.38;1.76)	1.73 (1.28;2.00)	0.652
lnRHI	0.44 (0.32;0.63)	0.42 (0.32;0.57)	0.55 (0.25;0.70)	0.652
Medical history				
Atrial fibrillation, n (%)	41 (56.2)	27 (51.9)	14 (66.7)	0.250
Hypertension, n (%)	67 (91.8)	48 (92.3)	19 (90.5)	1.000
Hyperlipidaemia, n (%)	47 (64.4)	33 (63.5)	14 (66.7)	0.796
Diabetes mellitus, n (%)	29 (39.7)	23 (44.2)	6 (28.6)	0.216
Coronary artery disease, n (%)	38 (52.1)	27 (51.9)	11 (52.4)	0.972
Peripheral artery disease, n (%)	7 (9.6)	6 (11.5)	1 (4.8)	0.665
Stroke/TIA, n (%)	17 (23.3)	16 (30.8)	1 (4.8)	0.017
Valve interventions, n (%)	12 (16.4)	11 (21.2)	1 (4.8)	0.160
Smoker active or ex-smoker, n (%)	38 (52.1)	27 (51.9)	11 (52.4)	0.972
COPD, n (%)	5 (6.8)	4 (7.7)	1 (4.8)	1.000
Sleep apnoea syndrome, n (%)	15 (20.5)	13 (25.0)	2 (9.5)	0.204
Obesity, n (%)	21 (28.8)	17 (32.7)	4 (19.0)	0.244
Anaemia, n (%)	19 (26.0)	16 (30.8)	3 (14.3)	0.146
eGFR (mL/min/1.73 m^2)	66.7 ± 18.6	64.2 ± 16.6	72.9 ± 22.1	0.070
Signs and symptoms				
NYHA class II, n (%)	58 (79.5)	39 (75.0)	19 (90.5)	0.204
NYHA class III, n (%)	15 (20.5)	13 (25.0)	2 (9.5)	0.204
Peripheral oedema, n (%)	24 (32.9)	18 (34.6)	6 (28.6)	0.785
Orthopnoea, n (%)	10 (13.7)	8 (15.4)	2 (9.5)	0.714
Fatigue, n (%)	42 (57.5)	31 (59.6)	11 (52.4)	0.609
Nocturnal cough, n (%)	7 (9.6)	7 (13.5)	0 (0.0)	0.182
Nocturia, n (%)	55 (75.3)	37 (71.2)	18 (85.7)	0.241
Medication				
ACE inhibitors or ARBs, n (%)	56 (76.7)	41 (80.8)	14 (66.7)	0.229
Beta-blockers, n (%)	61 (83.6)	42 (80.8)	19 (90.5)	0.489
Calcium antagonists, n (%)	30 (41.1)	22 (42.3)	8 (38.1)	0.741
Diuretics, n (%)	48 (65.2)	36 (69.2)	12 (57.1)	0.325
Aldosterone antagonists, n (%)	14 (19.2)	10 (19.2)	4 (19.0)	1.000
Echocardiography				
LAVI (mL/m^2)	42 (34;53)	47 (38;58)	33 (26;38)	<0.001
LVEDD (mm)	48.4 ± 6.5	48.9 ± 6.8	47.4 ± 5.8	0.383
IVSED (mm)	12.0 (11.0;13.0)	12.0 (11.0;13.0)	12.0 (10.5;13.5)	0.853
PWED (mm)	11.0 (10.0;12.0)	11.0 (10.0;12.0)	10.0 (9.0;12.0)	0.216
RWT	0.43 (0.38;0.51)	0.45 (0.38;0.52)	0.43 (0.38;0.49)	0.583
LVMI (g/m^2)	112 (85;127)	115 (90;128)	102 (75;126)	0.087
LVEF (%)	57 (55;60)	57 (55;60)	56 (55;60)	0.936
E (cm/s)	84 (68;104)	85 (67;104)	80 (68;104)	0.643
A (cm/s) (n = 51)	76 ± 26	77 ± 28	74 ± 22	0.686
E/A (n = 51)	1.0 (0.8;1.4)	1.0 (0.7;1.4)	1.0 (0.8;1.5)	0.536
e′ mean (cm/s)	6.7 ± 1.7	6.8 ± 1.6	6.6 ± 1.8	0.632
E/e′ mean	12.2 (10.2;16.6)	12.0 (10.2;15.3)	13.4 (10.2;17.1)	0.692
CPET and 6MWT				
Maximum workload (W)	90 (70;110)	90 (70;100)	90 (80;125)	0.216
Heart rate at rest (b.p.m.)	68 (63;77)	67 (63;75)	70 (59;81)	0.403
Exercise maximal heart rate (b.p.m.)	112 ± 28	112 ± 28	112 ± 28	0.993
Maximum RER	1.16 (1.10;1.24)	1.16 (1.09;1.22)	1.16 (1.12;1.27)	0.571
VE/VCO_2 Slope	35 (30;38)	36 (31;39)	34 (29;35)	0.108
PeakVO_2 (mL/kg/min)	15.3 (12.9;18.8)	15.3 (12.5;19.0)	15.9 (13.5;17.3)	0.534
Borg score CPET (6–20)	15 (15;17)	15 (15;17)	15 (15;17)	0.930
6MWT walk distance (m)	440 ± 100	427 ± 100	473 ± 92	0.073
Borg score 6MWT (6–20)	12 ± 3	12 ± 3	12 ± 2	0.639

6MWT, 6 min walk test; ACE, angiotensin-converting enzyme; ARBs, angiotensin receptor blockers; COPD, chronic obstructive pulmonary disease; CPET, cardiopulmonary exercise testing; E/e′ mean, left ventricular filling index; e′ mean, mean mitral annulus velocity; eGFR, estimated glomerular filtration rate; HFpEF, heart failure with preserved ejection fraction; IVSED, end-diastolic interventricular septal thickness; LAVI, left atrial volume index; lnRHI, natural logarithm of the reactive hyperaemia index; LVEDD, left ventricular end-diastolic

DOI: 10.1002/ehf2.14116

diameter; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass index; NYHA, New York Heart Association; peakVO_2, peak oxygen uptake; PWED, end-diastolic posterior wall thickness; RER, respiratory exchange ratio; RHI, reactive hyperaemia index; RWT, relative wall thickness; TIA, transient ischaemic attack; VE/VCO_2 Slope, exercise ventilatory efficiency.

1.67 ± 0.55 μmol/L for hArg, 0.74 (0.60;0.85) μmol/L for SDMA, 0.61 ± 0.10 μmol/L for ADMA, 207 ± 52 for the derived L-Arg/ADMA-Ratio, 2.64 (2.29;3.63) pg/mL for ET-1, 7.77 (4.69;18.96) pmol/L for copeptin, 208 (188;241) pg/mL for MR-proADM, 477 (272;949) pg/mL for NT-proBNP, and 1.8 (0.9;3.3) mg/L for hsCRP. Patients with definitive HFpEF tended to have higher ET-1 concentrations (P = 0.055). However, the concentrations did not differ significantly between the HFA-PEFF score groups. The copeptin and NT-proBNP concentrations were significantly higher in patients with definitive HFpEF (P = 0.044; $P < 0.001$). The median RHI in our study population was 1.55 (1.38;1.87), and the corresponding lnRHI 0.44 (0.32;0.63). Patients with definitive HFpEF had an RHI and lnRHI of 1.52 (1.38;1.76) and 0.42 (0.32;0.57), and patients at risk for HFpEF an RHI and lnRHI of 1.73 (1.28;2.00) and 0.55 (0.25;0.70). The difference was not statistically significant (P = 0.652). According to RHI cut-off values, 45 (61.6%) of our patients showed ED (RHI $\leq$ 1.67).

Correlations between biomarkers

Bivariate correlation analysis revealed a negative correlation of hArg with NT-proBNP (r = −0.275; P = 0.019), whereas SDMA positively correlated with NT-proBNP (r = 0.291; P = 0.013). SDMA also correlated with ET-1 (r = 0.233; P = 0.047) and copeptin (r = 0.381; P = 0.001), but not with MR-proADM or hsCRP. ADMA showed a positive correlation with ET-1 (r = 0.250; P = 0.033) and hsCRP (r = 0.303; P = 0.009) but did not correlate with NT-proBNP, copeptin, or MR-proADM. A table with all identified correlations between the markers can be found in the supporting information (*Table S1*).

Biomarkers and clinical characteristics

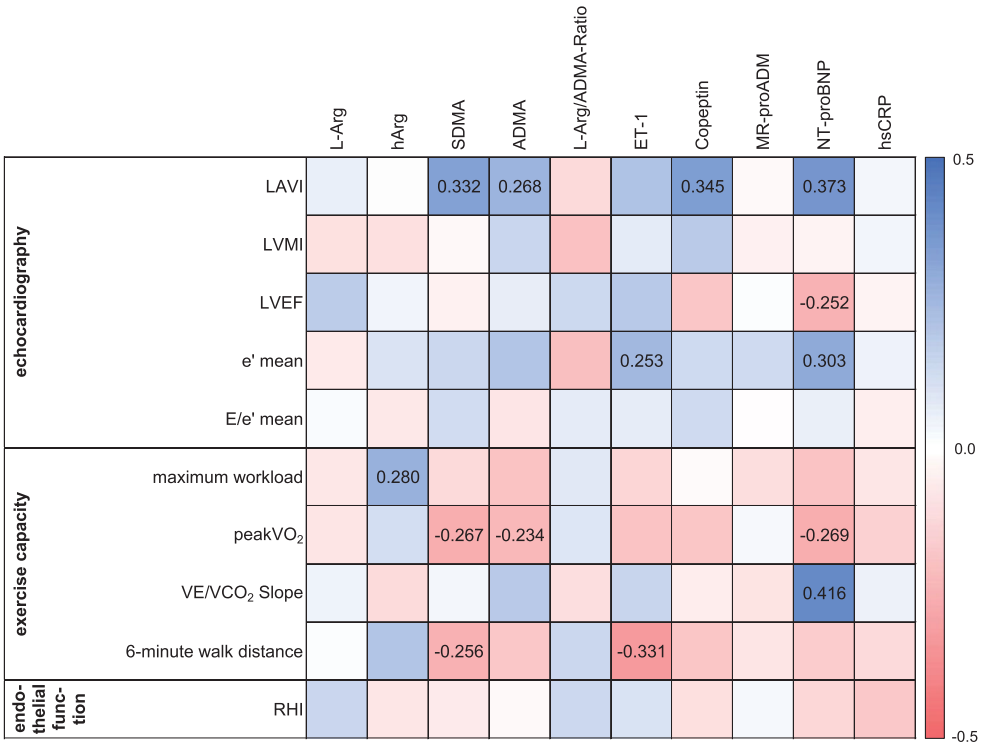
Results of the simple linear regression analysis (Model 1) between the investigated biomarkers and parameters of echocardiography, exercise capacity, and endothelial function are shown in *Figure 2*. Although some associations were found between biomarkers and parameters of echocardiography and exercise capacity, none of the markers were associated with the endothelial function reflected by RHI. For significant associations found in Model 1, a multiple linear regression analysis with adjustment for age and sex (Model 2) and age, sex, and comorbidities (Model 3) was performed (see Supporting Information, *Figures S1* and *S2*). SDMA was positively associated with the LAVI (β = 0.332; P = 0.004), and the association remained significant after adjustment for age and sex (Model 2) and further adjustment for BMI, AFib, eGFR, and hb (Model 3). In contrast, the association between ADMA and LAVI (β = 0.268; P = 0.022) was only significant prior to adjustment. Copeptin and NT-proBNP were also associated with LAVI across all three models (β = 0.345; P = 0.003 and β = 0.373; P = 0.001). In addition, NT-proBNP was the only marker being associated with the LVEF (β = −0.252; P = 0.032). After adjustment, the association was no longer significant. ET-1 and NT-proBNP were also associated with e' mean, with none of the two markers showing a significant association across all three models. Regarding exercise capacity, hArg correlated with maximum workload (β = 0.280; P = 0.016) in Model 1, but not after further adjustment. SDMA, ADMA,

Table 2 Biomarker concentrations

	All patients (n = 73)	HFpEF (n = 52)	Risk for HFpEF (n = 21)	P-value
L-Arg (μmol/L)	126 ± 32	128 ± 34	124 ± 29	0.614
Homoarginine (μmol/L)	1.67 ± 0.55	1.66 ± 0.58	1.68 ± 0.50	0.855
SDMA (μmol/L)	0.74 (0.60;0.85)	0.76 (0.61;0.87)	0.71 (0.58;0.78)	0.192
ADMA (μmol/L)	0.61 ± 0.10	0.63 ± 0.10	0.60 ± 0.11	0.318
L-Arg/ADMA-Ratio	207 ± 52	207 ± 55	208 ± 46	0.944
Endothelin-1 (pg/mL)	2.64 (2.29;3.63)	2.74 (2.31;3.71)	2.40 (2.16;3.02)	0.055
Copeptin (pmol/L)	7.77 (4.69;18.96)	9.60 (5.58;22.36)	5.04 (3.86;11.58)	0.044
MR-proADM (pg/mL)	208 (188;241)	214 (185;236)	200 (188;282)	0.738
NT-proBNP (pg/mL)	477 (272;949)	654 (335;1058)	253 (195;410)	<0.001
hsCRP (mg/L)	1.8 (0.9;3.3)	1.9 (0.8;3.6)	1.5 (0.9;2.6)	0.393

ADMA, asymmetric dimethylarginine; HFpEF, heart failure with preserved ejection fraction; hsCRP, high-sensitivity C-reactive protein; L-Arg, L-arginine; MR-proADM, mid-regional fragment of pro-adrenomedullin; NT-proBNP, N-terminal pro-B-type natriuretic peptide; SDMA, symmetric dimethylarginine.

Figure 2 Heatmap for the simple linear regression analysis between biomarkers and clinical characteristics (Model 1). Scale for the regression coefficient β from 0.5 to −0.5; in cases of significance (*P*-value < 0.05), β is given in the corresponding field. ADMA, asymmetric dimethylarginine; E/e' mean, left ventricular filling index; e' mean, mean early diastolic mitral annulus velocity; ET-1, endothelin-1; hArg, homoarginine; hsCRP, high-sensitivity C-reactive protein; L-Arg, L-arginine; LAVI, left atrial volume index; LVEF, left ventricular ejection fraction; LVMI, left ventricular mass index; MR-proADM, mid-regional pro-adrenomedullin; NT-proBNP, N-terminal pro-brain natriuretic peptide; peakVO_2, peak oxygen uptake; RHI, reactive hyperaemia index; SDMA, symmetric dimethylarginine; VE/VCO_2 Slope, exercise ventilatory efficiency.



and NT-proBNP were negatively associated with peakVO_2 (β = −0.267; *P* = 0.022, β = −0.234; *P* = 0.046, and β = −0.269; *P* = 0.021). Adjustment, however, led to a loss of significance. In contrast, the association between NT-proBNP and VE/VCO_2 Slope (β = 0.416; *P* < 0.001) remained significant across all three models. SDMA and ET-1 were inversely associated with 6MWT distance (β = −0.256; *P* = 0.029 and β = −0.331; *P* = 0.004). After adjustment in Models 2 and 3, there was a loss of significance for the association of SDMA and 6MWT distance. For ET-1, the association remained significant after adjustment for age and sex but not after further adjustment in Model 3.

Principal component analysis

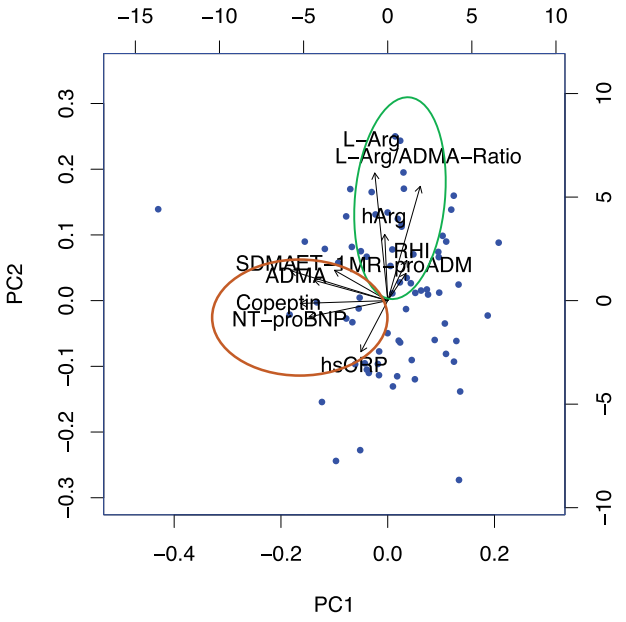
Two principal components were retained. Principal components 1 (PC1) and 2 (PC2) explained 23.46% and 18.68% of the variance, respectively. The PCA revealed two clusters (see *Figure 3*). In particular, ADMA, SDMA, ET-1, copeptin, NT-proBNP, and hsCRP mainly loaded in PC1, whereas L-Arg, the L-Arg/ADMA-Ratio, hArg, MR-proADM, and the RHI mainly loaded in PC2.

Discussion

In a cohort of well-characterized HFpEF patients, we found that (i) markers of NO metabolism correlated with the established HF biomarker NT-proBNP as well as biomarkers reflecting inflammation and neurohumoral activation; (ii) SDMA, a methylated arginine derivative that affects NO synthesis by lowering substrate availability, was associated with the LAVI, also after adjustment for age, sex, and comorbidities; and (iii) in PCA, two biomarker clusters could be derived.

To describe the characteristics of our cohort, we divided our study population in two groups based on retrospectively calculated HFA-PEFF scores. Seventy-one per cent of our patients could be defined as patients with definitive HFpEF (scores 5–6), and 29% as patients with risk for HFpEF (scores 3–4). This distribution is comparable with recent studies that assessed the HFA-PEFF score in existing HFpEF cohorts.[35,36] Although clinical characteristics did not significantly differ between both groups, patients with definitive HFpEF showed significant differences in the LAVI and the established HF biomarker NT-proBNP. This suggests that deeper phenotypization is important for clinical assessment of HFpEF patients. Regarding the investigated L-Arg/NO metabolites,

Figure 3 Principal component analysis from the investigated biomarkers and the reactive hyperaemia index (RHI). Principal component 1 (PC1) and principal component 2 (PC2) explain 23.46% and 18.68% of the variance. ADMA, asymmetric dimethylarginine; ET-1, endothelin-1; hArg, homoarginine; hsCRP, high-sensitivity C-reactive protein; L-Arg, L-arginine; MR-proADM, mid-regional pro-adrenomedullin; NT-proBNP, N-terminal pro-brain natriuretic peptide; SDMA, symmetric dimethylarginine.



we could not identify statistically different concentrations between patients with definitive HFpEF and patients at risk. A possible explanation is that patients at risk for HFpEF suffer from comparable abnormalities in the NO metabolism as patients with definitive HFpEF, which seems plausible, especially considering the aspect of an impaired NO metabolism as possible key pathogenic determinant particularly in the development of the disease.[3] Moreover, it should be recalled that, although patients were categorized in the two groups 'definitive HFpEF' and 'risk for HFpEF', all patients included in our analysis were patients with HFpEF among criteria of the ESC HF guidelines 2016. This might be a reason for rather similar concentrations of the L-Arg/NO metabolites between the two groups.

We found that SDMA positively and hArg negatively correlated with NT-proBNP, as previously described by Pilz *et al*. in a large multicentre study including 1396 primary care patients at cardiovascular risk with preserved LVEF.[37] Moreover, we demonstrated a positive correlation of both ADMA and SDMA with ET-1. ED is not only acknowledged as the lower bioavailability of NO but also a failure of the physiological balance between NO and endothelin.[38] Thus, this finding supports the hypothesis that methylarginines are elevated in states of ED in patients with HFpEF. AVP, in laboratory analysis reflected by copeptin, is supposed to have profibrotic effects by promoting proliferation and differentiation of cardiac fibroblasts.[39] This hypothesis may be supported by our finding that copeptin was positively associated with LAVI. Accordingly, the positive correlation of SDMA with copeptin may reflect the idea that SDMA contributes to profibrotic cardiac remodelling processes. In line with this idea, Schepers *et al*. found SDMA as proinflammatory agent that increases the production of interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα).[40] Moreover, SDMA was described to increase the generation of reactive oxygen species (ROS).[41] IL-6, TNFα, and ROS are supposed to contribute to profibrotic cardiac remodelling processes in HFpEF.[3] Our finding of a positive correlation between ADMA and hsCRP is consistent with the proposed concept of a systemic proinflammatory state that leads to an impairment of the NO pathway in patients with HFpEF.[3]

Recently, Hage *et al*. reported on higher SDMA [0.5 (0.4;0.6) μmol/L] and ADMA levels [0.6 (0.5;0.6) μmol/L] in patients with HFpEF compared with healthy controls. Moreover, they found higher SDMA levels in patients with diastolic dysfunction ($E/e' > 14$ vs. $E/e' \leq 14$; $P = 0.039$).[42] We demonstrated similar ADMA and higher SDMA levels in our cohort but did not identify associations of SDMA and diastolic function in our linear regression analysis. However, we found significant associations of both ADMA and SDMA and LAVI and, thus, further support for the hypothesis that methylarginines are involved in structural heart disease. It should be noted that only the association of SDMA and LAVI remained significant after adjustment for other variables, whereas the association of ADMA and LAVI was lost after adjustment.

Maréchaux *et al*. compared the endothelial function in patients with HFpEF and patients with hypertension but no history of HF and found that patients with HFpEF had a significantly lower brachial artery FMD and therefore depressed endothelial function.[10] Akiyama *et al*. described a significantly lower lnRHI in patients with HFpEF compared with patients without HFpEF (0.53 ± 0.20 vs. 0.64 ± 0.20; $P < 0.001$).[11] In our cohort of HFpEF patients, we found an overall RHI and lnRHI of 1.55 (1.38;1.87) and 0.44 (0.32;0.63). Although ED is closely linked to reduced NO bioavailability, we identified no significant correlations between the RHI and markers of NO metabolism.[9] This raises the question whether the RHI ideally reflects NO bioavailability. It should be noted that, to date, the physiology of PAT still remains unclear.[9] PAT measures microvessel dilatation in the highly complex digital vasculature, which consists of nutritive vessels and arteriovenous anastomoses.[43] The resting vascular tone of the anastomoses is mainly regulated by the sympathetic nervous system and is thus only partly dependent on NO.[43] Accordingly, it may be concluded that the augmentation of the arterial pulse volume amplitude after reactive hyperaemia involves mechanisms that are both related and unrelated to the endothelium, with a limited specificity as a measure of NO bioavailability.[43,44] Combining this aspect with

our finding on no significant correlations between the RHI and markers of NO metabolism, we have to conclude that the RHI may be non-reflective of NO-dependent processes that are proposed to contribute to the development of cardiac hypertrophy and increased cardiomyocyte resting tension in patients with HFpEF. In contrast, in patients that were referred to a hospital for coronary angiography, Bonetti *et al*. found a significantly lower RHI in patients with coronary ED compared with those with normal coronary endothelial function. Coronary endothelial function was assessed by intracoronary infusion of acetylcholine.[45]

Impaired exercise capacity is a significant predictor of morbidity and mortality in patients with HFpEF.[34] Consequently, improving exercise capacity is one of the major targets in HFpEF therapy studies. Several randomized controlled trials targeting the NO pathway, including inorganic nitrites, organic nitrates, and soluble guanyl cyclase stimulators, failed to improve exercise capacity in patients with HFpEF.[16–18] Possible reasons of the neutral results may be an inadequate administration of the therapeutic agents, a lack of targeted NO release and cGMP up-regulation during exercise, and tachyphylaxis.[46] However, a recent study argues for further evaluation of NO-based therapies in HFpEF. Reddy *et al*. demonstrated favourable combined pulmonary, cardiac, and peripheral effects of inorganic nitrite during exercise in HFpEF patients.[47] Regarding methylarginines like ADMA and SDMA, there is still a lack of potential therapeutic strategies in this field, although mechanisms on how methylated arginine derivatives affect NO synthesis are widely described. This might be due to the fact that much of our today's knowledge on potential therapies involving lowering ADMA and SDMA is based on animal research. Further studies are required to fill the translational gap between animal models and clinical trials.[19] It may be helpful to identify a subpopulation of HFpEF patients that is most likely to benefit from therapies targeting NO metabolism and pathway.

Our PCA revealed two clusters within our cohort. This finding highlights the aspect that there is probably no 'one size fits all' concept of HFpEF, neither in the pathophysiological understanding of the clinical syndrome nor in its therapy. The markers that mainly contributed to PC1 in the PCA, among them ADMA, SDMA, copeptin, and hsCRP, may represent the aspect of lower NO bioavailability, whereas hArg and the L-Arg/ADMA-Ratio that mainly contributed to PC2 may represent the aspect of a better NO bioavailability and a therefore contrary state.

Limitations

We reported on a relatively small single cohort with no long-term follow-up available yet. Thus, the results should be considered with caution. One limitation of our study is the retrospective assessment of the HFA-PEFF score. The absence of data on peak velocity of the tricuspid regurgitation and global longitudinal strain may have altered the calculation of the HFA-PEFF score. Analysing the biomarker serum concentrations, we did not divide our study cohort by sex. However, the gender ratio of our cohort was almost equal. There was no healthy control group available to compare the biomarker concentrations and RHI. With an explained total variance of 42.14% in the PCA, the conclusions of this analysis should also be considered with caution.

Conclusions

Our findings suggest an impaired NO metabolism as a possible key pathogenic determinant in at least a subgroup of patients with HFpEF. It would be of special interest to measure the concentrations of NO metabolites in upcoming HFpEF studies to clarify whether subgroups of patients can take more benefit from therapies that are targeting NO metabolism and pathway.

Acknowledgements

Open Access funding enabled and organized by Projekt DEAL. WOA Institution: Charite Universitatsmedizin Berlin Consortia Name : Projekt DEAL

Conflict of interest

K.P., A.F., V.Z., C.R., A.B., E.B., E.P., A.K., E.S., S.H., B.P., and F. E. declare that they have no conflict of interest.

Funding

This work was supported by the German Research Foundation (DFG).

Supporting information

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

Table S1. Results of bivariate correlation analysis between all investigated biomarkers (Spearman's correlation coefficient is given in each field; significant correlations are highlighted in bold; *$p < 0.05$; **$p < 0.01$).

Figure S1. Heatmap for the multiple linear regression analysis between biomarkers and clinical characteristics, adjusted for

age and sex (model 2). Scale for the regression coefficient β from 0.5 to −0.5; in cases of significance (p-value <0.05), β is given in the corresponding field. L-Arg, L-arginine; hArg, homoarginine; SDMA, symmetric dimethylarginine; ADMA, asymmetric dimethylarginine; ET-1, endothelin-1; MR-proADM, midregional proadrenomedullin; NT-proBNP, N-terminal pro brain natriuretic peptide; hsCRP, high sensitivity C-reactive protein; LAVI, left atrial volume index; LVMI, left ventricular mass index; LVEF, left ventricular ejection fraction; e' mean, mean early diastolic mitral annulus velocity; E/e' mean, left ventricular filling index; peakVO_2, peak oxygen uptake; VE/VCO_2 Slope, exercise ventilatory efficiency; RHI, reactive hyperaemia index.

Figure S2. Heatmap for the multiple linear regression analysis between biomarkers and clinical characteristics, adjusted for age, sex, body mass index, atrial fibrillation, estimated glomerular filtration rate and haemoglobin (model 3). Scale for the regression coefficient β from 0.5 to −0.5; in cases of significance (p-value <0.05), β is given in the corresponding field. L-Arg, L-arginine; hArg, homoarginine; SDMA, symmetric dimethylarginine; ADMA, asymmetric dimethylarginine; ET-1, endothelin-1; MR-proADM, midregional proadrenomedullin; NT-proBNP, N-terminal pro brain natriuretic peptide; hsCRP, high sensitivity C-reactive protein; LAVI, left atrial volume index; LVMI, left ventricular mass index; LVEF, left ventricular ejection fraction; e' mean, mean early diastolic mitral annulus velocity; E/e' mean, left ventricular filling index; peakVO_2, peak oxygen uptake; VE/VCO_2 Slope, exercise ventilatory efficiency; RHI, reactive hyperaemia index.

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