ORIGINAL RESEARCH ARTICLE

Metabolic profiles in heart failure due to non-ischemic cardiomyopathy at rest and under exercise

Matthias Mueller-Hennessen[1,5†], Johanna Sigl[1†], Jens C. Fuhrmann[3], Henning Witt[3], Regina Reszka[4], Oliver Schmitz[3], Jürgen Kastler[3], Jenny J. Fischer[3], Oliver J. Müller[1,5], Evangelos Giannitsis[1], Tanja Weis[1,5], Norbert Frey[2,5] and Hugo A. Katus[1,5]*

[1]*Department of Internal Medicine III, Cardiology, Angiology & Pneumology, University of Heidelberg, Heidelberg, Germany;* [2]*Department of Cardiology and Angiology, University of Kiel, Kiel, Germany;* [3]*metanomics GmbH, Berlin, Germany;* [4]*Metanomics Health GmbH, Berlin, Germany;* [5]*DZHK (German Centre for Cardiovascular Research), Berlin, Germany*

Abstract

Aims Identification of metabolic signatures in heart failure (HF) patients and evaluation of their diagnostic potential to discriminate HF patients from healthy controls during baseline and exercise conditions.
Methods Plasma samples were collected from 22 male HF patients with non-ischemic idiopathic cardiomyopathy and left ventricular systolic dysfunction and 19 healthy controls before (t0), at peak (t1) and 1 h after (t2) symptom-limited cardiopulmonary exercise testing. Two hundred fifty-two metabolites were quantified by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC)-MS/MS-based metabolite profiling.
Results Plasma metabolite profiles clearly differed between HF patients and controls at t0 ($P < 0.05$). The metabolic signature of HF was characterized by decreased levels of complex lipids and fatty acids, notably phosphatidylcholines, cholesterol, and sphingolipids. Moreover, reduced glutamine and increased glutamate plasma levels, significantly increased purine degradation products, as well as signs of impaired glucose metabolism were observed. The metabolic differences increased strongly according to New York Heart Association functional class and the addition of three metabolites further improved prediction of exercise capacity ($Q^2 = 0.24$ to 0.35). Despite a high number of metabolites changing significantly with exercise (30.2% at t1/t0), the number of significant alterations between HF and controls was almost unchanged at t_1 and t_2 (30.7 and 29.0% vs. 31.3% at t_0) with a similar predictive group separation ($Q^2 = 0.50$ for t0, 0.52 for t1, and 0.56 for t2, respectively).
Conclusions Our study identified a metabolic signature of non-ischemic HF with prominent changes in complex lipids including phosphatidylcholines, cholesterol, and sphingolipids. The metabolic changes were already evident at rest and largely preserved under exercise.

Introduction

Heart failure (HF) constitutes a major burden on the health care systems and projected demographics indicate a further increase.[1] Despite established criteria currently based on clinical presentation, imaging techniques, and natriuretic peptides,[2] the diagnosis and risk stratification of HF can be equivocal due to individual variations. Because HF is related to the inability of the heart to meet the body's metabolic demands resulting in major changes of the overall metabolism,[3] metabolic profiling is a promising approach to provide insights into pathogenesis and molecular pathways[4,5] as well as for the identification of new biomarkers.[6,7] Even though it is cost-consuming and time-consuming, cardiopulmonary exercise testing currently assists the functional diagnostic classification of HF and provides independent additional information on disease severity and associated risk.[8,9] Exercise physiology is well reflected in the blood and skeletal muscle

profiles of low-molecular-weight metabolites,[10,11] as confirmed by modern metabolite profiling techniques.[12] Therefore, addition of metabolic data to clinical characteristics may enhance their power for prediction of exercise capacity.

To identify novel biomarkers for HF, we thus analysed the metabolic profile in HF patients with a confirmed diagnosis of non-ischemic idiopathic cardiomyopathy (NICM) and left ventricular (LV) systolic dysfunction in comparison to an age-matched control group. In addition, we aimed to evaluate if the changes in HF-associated metabolic profiles are further increased during and after exercise, thereby unmasking metabolic compensation mechanisms at rest and possibly improving the power of discrimination. Broad untargeted profiling based on gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC)-MS/MS technologies was applied to ensure high coverage of the metabolite spectrum. Catecholamines and derivatives as well as steroids were selected as particularly relevant metabolite groups and quantified by targeted methods.

Methods

Patients and controls

Male patients (n = 22) with HF and LV systolic dysfunction due to NICM in New York Heart Association (NYHA) functional classes I (n = 6), II (n = 11) and III (n = 5) were enrolled. Exclusion criteria were female gender, age <18 or >75 years, LV-ejection fraction (EF) ≥50%, NYHA functional class IV, stroke or myocardial infarction within the last 4 months, angiographically confirmed significant coronary artery disease, surgery, or any modification of pharmacological therapy within the last month, reduced renal function (plasma creatinine >2 mg/dL), autoimmune diseases and malignant tumours. All patients were phenotyped based on cardiac catheterization including hemodynamic measurements. A control group of healthy men matched for age and BMI (n = 19) was included. All patients were recruited from the Department of Cardiology, University Hospital of Heidelberg, Germany. Controls were recruited from the employees of the University Hospital of Heidelberg. The Ethical Committee of the University of Heidelberg approved the study protocol, which was performed according to the principles of the Declaration of Helsinki, and informed consent was signed by all subjects.

Cardiopulmonary exercise testing

All patients and controls underwent symptom-limited cardiopulmonary exercise testing (Ergoline Ergometrics 900®, VIASYS Healthcare GmbH). Starting from a work-load of 15 Watts, power was increased by 15 W every 2 min. ECG and blood pressure were documented before, continuously during and after exercise. Criteria for premature termination were chest pain, severe dyspnea, severe arrhythmias, ST segment depression or elevation >0.2 mV, or a drop in systolic blood pressure of >20 mmHg during exercise.

Imaging techniques

Echocardiography was performed in all 22 HF patients and LV-EF was determined by the biplane disc summation method (Simpson rule). In the control group, 15 subjects (79%) underwent Gadolinium contrast-enhanced cardiac magnetic resonance imaging (cMRI) including dobutamine stress testing, and four subjects (21%) underwent 2-D echocardiography at rest and under bicycle stress-echocardiography for quantification of LV-EF, and to exclude underlying significant coronary heart disease.

Blood sampling

Blood was collected from all patients and controls following 5 min of rest in supine position directly before (t0), at the peak (t1), and 1 h after termination (t2) of cardiopulmonary exercise testing. Subjects were fasted for at least 8 h to minimize the influence of nutrition on metabolite levels. High sample quality and reliable results were ensured by pre-analytics following a fixed procedure as a standard operating protocol. Blood was collected in EDTA Monovette® tubes (Sarstedt AG&Co, Germany) which were gently inverted five times to allow mixing. Samples were stored for a maximum of 1 h at 4°C before centrifugation at 1000 **g** for 10 min. Plasma was immediately transferred to a prelabeled 1 mL Mikro tube (Sarstedt AG & Co, Germany) and stored at −80°C until shipment on dry ice.

Metabolite profiling

Broad metabolite profiling as well as quantification of catecholamines and steroids from human plasma samples were performed at metanomics GmbH (Berlin, Germany) as previously described.[13,14] Briefly, MxP® Broad profiling was performed using gas chromatography-mass spectrometry (Agilent 6890 GC coupled to an Agilent 5973 MS-System) and LC-MS/MS (Agilent 1100 HPLC-System coupled to a MS/MS-System from Applied Biosystems API4000). For determination of catecholamines and steroids, solid phase extraction (SPE; Spark Symbiosis Pharma) coupled to LC-MS/MS was used.

Statistical analysis

Distributions of the clinical characteristics were analysed by Shapiro–Wilk test. Parameters following normal distribution

were compared by Student's *t*-test, and parameters deviating from normal distribution were analysed by Kruskal–Wallis test using SPSS 16.0 for Windows.

Multivariate analysis including principal component analysis (PCA) and partial least squares -discriminant analysis (PLS-DA) was applied using Umetrics SIMCA P+ 12 (MKS Data Analytics Solutions). All data were log-transformed, centred, and scaled to unit variance. The cross-validated cumulative Q^2 value was used to assess the predictive value of the PLS-DA model. Univariate analysis was performed by repeated-measures ANOVA using R-software package nlme, details are given in the Supporting Information.

Results

Clinical characteristics

Clinical characteristics of HF patients due to NICM and the healthy control group are presented in *Table 1*. In HF patients, cardiopulmonary exercise performance was significantly reduced compared with healthy controls (*Table 1*).

Overview of the metabolic dataset

In plasma samples from patients and healthy controls, a total of 252 metabolites fulfilled quality criteria for quantification. Of these, 164 could be precisely assigned to known metabolites, while 88 analytes required additional structure elucidation efforts and were therefore classified as 'unknown'. Data for 250 metabolites (including unknown metabolites) were

used for analysis (two metabolites were excluded due to missing data). The distribution of the metabolites across major biochemical groups is shown in *Figure 1*. Supervised PLS-DA analysis showed a complete separation of the groups ($Q^2_{cumulative}$ = 0.52) and identified drivers of the separation according to NYHA class and metabolite ontology group (*Figure 2*). Comparison of metabolite profiles of the entire cohort at baseline (t0) to peak-exercise (t1) by PLS-DA ($Q^2_{cumulative}$ = 0.39) (Supporting Information *Figure S1*) also indicated a marked impact of exercise on a subset of plasma metabolite levels. In addition, the impact of both disease and exercise on metabolite levels was clearly demonstrated by repeated-measures ANOVA (*Table 2*).

Baseline differences of the metabolic profile

A total of 79 metabolites (31.3%) showed significantly different baseline values ($P < 0.05$) between HF patients and controls. The metabolite classes most strongly affected in HF due to NICM compared with controls at t0 were complex lipids, fatty acids, and related [33 of 60 metabolites of the class significantly different with reduced phosphatidylcholines (PC), lysophosphatidylcholines (LPC), cholesterol, sphingolipids, free and lipid-bound fatty acids], amino acids and derivatives (10/33, including elevated glutamate, alpha-ketoglutarate, and 2-hydroxybutyrate and reduced histidine), hormones, signal substances and related (9/23, among them elevated noradrenaline and normetanephrine), carbohydrates and related (4/11, with elevated mannose, glucose and reduced 1,5-anhydrosorbitol) and nucleobases and related (3/3, with elevated hypoxanthine, and pseudouridine). Metabolites with most pronounced baseline differences between HF patients and controls are shown in

Table 1 Clinical characteristics of HF patients due to non-ischemic cardiomyopathy and controls

Characteristics	Controls (*n* = 19)	HF patients (*n* = 22)	*P*-value
Male gender, n (%)	19 (100)	22 (100)	NA
Age [years] (±SD)	47.5 (±12.8)	51.5 (±13.7)	0.34
BMI [kg/m^2] (± SD)	25 (±3)	28 (±4)	0.45
NYHA class I, II, III, n (%)	0	6 (27), 11 (50), 5 (23)	NA
LV-EF 1, n (%)	19 (100)	0	NA
LV-EF 2, 3, 4, n (%)	0	5(23), 4(18), 13 (59)	NA
LV-EF [%]	57 (±4)	29 (±14)	<0.0001
eGFR [mL/min] (±SD)	118.7 (±29.2)	115.6 (±43.5)	0.79
Arterial hypertension, *n* (%)	0	12 (55)	NA
Hypercholesterolaemia, *n* (%)	0	9 (41)	NA
Diabetes mellitus, *n* (%)	0	5 (23)	NA
Ex/smoker, *n* (%)	0	10/3 (45/14)	NA
BP at rest [mmHg] (±SD)	128 (±12)	117 (±22)	0.057
BP at max. exercise [mmHg] (±SD)	188 (±12)	163 (±32)	0.002
Power output [W] (±SD)	182 (±43)	114 (±46)	<0.001
VO$_{2,max}$ [mL O$_2$/min] (±SD)	32 (±6)	21 (±8)	<0.001

SD, standard deviation; BMI, body mass index; NYHA, New York Heart Association; LV-EF 1, left ventricular ejection fraction >55%; LV-EF 2, 45-55%; LV-EF 3, 31-44%; LV-EF 4, <31%; eGFR, estimated glomerular filtration rate; BP, systolic blood pressure; VO$_{2,max,}$ max. oxygen uptake during exercise
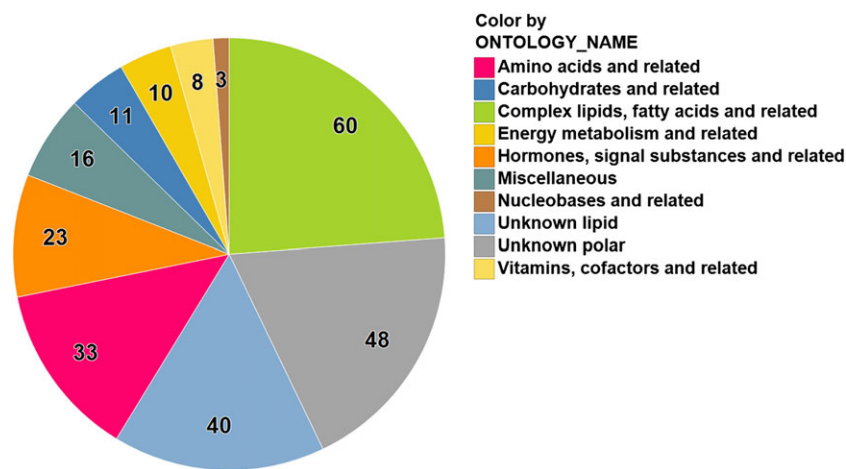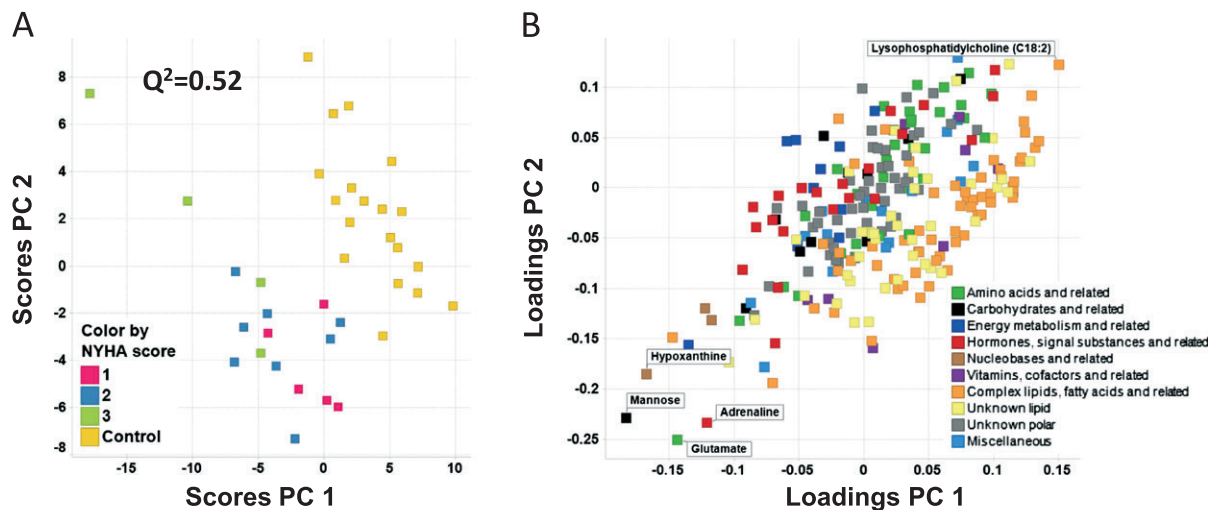
Figure 1 Distribution of metabolites across major biochemical groups.

Figure 2 PLS-DA scores plot showing group separation between HF patients due to non-ischemic cardiomyopathy and controls at rest (t_0) colored by NYHA class (A) and loadings plot colored by metabolite ontology group (B).

Table 3. The baseline differences after additional correction for diabetes and estimated glomerular filtration rate (eGFR) can be found in *Table S1*. Moreover, Bonferroni correction for multiple testing ($P < 0.000198$) revealed significant differences for mannose (elevated 1.9-fold of control levels), LPC (C18:2) (reduced to 0.6 of control levels), hypoxanthine (elevated 2.1-fold), phytosphingosine (reduced to 0.8), lignoceric acid (C24:0) (reduced to 0.7), glutamate (elevated 2.0-fold), and 2-hydroxybutyrate (elevated 1.7-fold). Analysis of metabolic data in relation to LV-EF or NYHA functional class by ANOVA revealed a clear correlation of the metabolic changes with clinical HF stage and severity. The percentage of significant changes rose from

7.6% in NYHA I to 22.3% in NYHA II and 37.1% in NYHA III (*Table 2*). Similar results were observed when metabolite profiling data were correlated with LV-EF (*Table 2*).

Differentiation of non-ischemic HF and controls using metabolic profiling during exercise

Overall, the metabolites and the direction of change was conserved because the numbers of significantly different metabolites between HF and controls changed only slightly from 79 (31.3%) at baseline to 76 at t1 (30.7%) and 73 at t2 (29.0%)

Table 2 Overview of ANOVA significances depending on clinical HF diagnosis, LV systolic function and NYHA functional class

| Diagnosis criteria | HF | LV-EF | NYHA (categorical) | | |
| | | (numerical) | NYHA 1 | NYHA 2 | NYHA 3 |
Factor	% significances	% significances		% significances	
Age	19.0	17.5	21.1		
BMI	12.3	13.9	9.6		
Time t1/t0	30.2	30.2	29.5		
Time t2/t0	24.2	24.2	23.5		
Time t2/t1	40.6	40.6	38.9		
Diagnosis at t0	31.3	32.1	7.6	22.3	37.1
Diagnosis at t1	30.7	30.6	6.7	22.2	36.1
Diagnosis at t2	29.0	31.7	7.9	23.0	35.3
Interaction Time (t1/t0):Diagnosis	6.0	9.1	2.0	4.8	4.4
Interaction Time (t2/t0):Diagnosis	9.5	10.3	0.8	4.8	10.0
Interaction Time (t2/t1):Diagnosis	10.0	9.5	0.8	6.3	11.1

BMI, body mass index; HF, heart failure; NICM, non-ischemic cardiomyopathy; LV-EF, left ventricular ejection fraction; NYHA, New York Heart Association.

(*Table 2*). Multivariate analysis confirmed this finding showing a similar predictive group separation between HF patients and controls at all three time points ($Q^2_{cumulative}$ = 0.50 for t0, 0.52 for t1, and 0.56 for t2, respectively). Analysis of metabolite profiles at the peak of exercise (t1) added further three significantly alterered metabolites inpatients compared with controls: lactate, citrate, and one unknown metabolite.

Comparison of the exercise response between non-ischemic HF and controls

Figure 3A shows a comparison of the exercise response (t1 vs. t0) between HF and controls. When comparing baseline to peak of exercise (t1/t0), significant changes were observed in 81 (32.1%) metabolites for HF patients and in 74 (29.4%) metabolites for healthy controls. A list of metabolites with significant exercise-induced changes (*P* < 0.05) is given in *Table S2*. Sixty-six metabolites showed significantly changed levels in both HF patients and controls with identical direction of regulation in all metabolites. In contrast, eight metabolites were only significantly changed in controls and 15 metabolites showed a significant exercise-induced change only in HF patients including glutamate, threonic acid, PC (C18:0/C20:4), LPC (C18:1), LPC (C17:0), and coenzyme Q9 (*Table S2*). By ANOVA analysis of the interaction between diagnosis and exercise (t1/t0), 13 metabolites showed *P* < 0.05 (*Table 2*). Among these, lactate, adrenaline, hypoxanthine, glutamate, and indol-3-lactic acid had *P* < 0.01. *Figure 3B* displays the pattern of regulation for lactate and adrenaline during exercise. After correction for exercise performance, only changes in glutamate levels remained significantly different between HF and controls (*P* < 0.05).

Correlation of metabolites to exercise capacity and predictive value

The most significant correlations of metabolites with maximal oxygen uptake ($VO_{2,max}$) are summarized in *Table S3A*. Metabolites from the ontology classes of complex lipids and energy metabolism had the highest frequency of significant correlations with $VO_{2,max}$, which were relatively consistent over different time intervals (*Table S3B*). In total, 79 metabolites (31.9%) showed a significant correlation to $VO_{2,max}$ at t0, 78 (31.5%) at t1, and 64 (25.8%) at t2, respectively.

For prediction of $VO_{2,max}$ by PLS-DA analysis, NYHA, NT-proBNP, or LV-EF alone showed only weak Q2-values at baseline (t0) (*Table S3C*). Using only one metabolite [erythro-sphingosine (d18:1)], a similar prediction power could be observed in comparison to NYHA functional class ($Q^2_{cumulative}$ = 0.25 vs. 0.24) and use of three metabolites [erythro-sphingosine, LPC (C17:0), LPC (C20:4); ($Q^2_{cumulative}$ = 0.25 vs. 0.26)]. However, the combination of NYHA and all three metabolites at baseline showed a higher Q2-value of 0.35, which was similar to the results obtained when using NYHA and five metabolites. At t1, again the combination of NYHA and three metabolites [cholestenol No. 02, sphingomyelin (d18:2,C16:0), lactate] exhibited the best performance for prediction of $VO_{2,max}$ ($Q^2_{cumulative}$ = 0.40) (*Table S2*).

Discussion

In this study, plasma metabolite profiles were evaluated in HF patients with LV systolic dysfunction due to NICM in comparison to healthy controls. Patients and controls

Table 3 List of metabolites significantly changed in HF due to non-ischemic cardiomyopathy vs. controls at baseline

Ontology	Metabolite	Contrast — All patients vs. Control at t0 Ratio	NYHA 1 vs. Control at t0 Ratio	NYHA 2 vs. Control at t0 Ratio	NYHA 3 vs. Control at t0 Ratio	NYHA (numerical) at t0 Ratio	LVEF at t0 Ratio
Amino acids and related	Glutamate	1.80	1.48	**2.02**	1.83	1.33	0.78
	Histidine	0.82	0.97	0.74	0.83	0.90	1.11
	alpha-Ketoglutarate	1.45	1.11	1.37	**2.24**	1.28	0.80
Carbohydrates and related	Mannose	1.87	1.76	1.74	1.56	1.29	0.81
Complex lipids, fatty acids and related	Cholesterol	0.82	0.96	0.83	0.68	0.88	1.11
	Behenic acid (C22:0)	0.71	0.90	0.76	0.53	0.81	1.17
	Lignoceric acid (C24:0)	0.67	0.84	0.68	**0.50**	0.79	1.24
	Linoleic acid (C18:cis[9,12]2)	0.72	0.83	0.70	0.64	0.83	1.18
	Tricosanoic acid (C23:0)	0.67	0.85	0.71	**0.46**	0.78	1.21
	Galactose, lipid fraction	0.81	0.88	0.81	0.74	0.89	1.09
	Glycerol phosphate, lipid fraction	0.70	0.85	0.70	0.58	0.82	1.22
	Lysophosphatidylcholine (C17:0)	0.74	0.87	0.76	0.71	0.85	1.15
	Lysophosphatidylcholine (C18:0)	0.79	0.92	0.80	0.76	0.88	1.16
	Lysophosphatidylcholine (C18:1)	0.86	0.92	0.92	0.83	0.92	1.09
	Lysophosphatidylcholine (C18:2)	0.72	0.74	0.76	0.63	0.85	1.22
	Phosphate, lipid fraction	0.80	0.90	0.83	0.70	0.89	1.11
	Phosphatidylcholine (C16:1, C18:2)	0.82	0.86	0.85	0.72	0.90	1.12
	5-O-Methylsphingosine (3 additionals)	0.76	0.93	0.77	0.58	0.83	1.16
	erythro-Sphingosine (1 additional)	0.78	0.94	0.79	0.61	0.85	1.14
	Phytosphingosine	0.72	0.88	0.66	0.68	0.84	1.15
Energy metabolism and related	2-Hydroxybutyrate	1.57	1.46	1.57	1.71	1.24	0.86
Nucleobases and related	Hypoxanthine (1 additional)	**2.09**	1.87	1.94	**2.74**	1.45	0.73
	Pseudouridine	1.42	1.14	1.25	**2.05**	1.25	0.81
Unknown lipid	Unknown lipid A	0.75	0.87	0.74	0.66	0.85	1.16
	Unknown lipid B	0.59	0.75	0.68	**0.47**	0.75	1.37
	Unknown lipid C	0.77	0.91	0.79	0.72	0.87	1.16

ANOVA results filtered by P-value and sorted by ontology class in the comparison of HF vs. control at t0 ($P < 0.001$). The results for invidual NYHA classes and the correlation with LV-EF are shown for comparison. Ratio columns show the direction and strength of regulation (estimated ratio of group averages from ANOVA). Intensities of the colors indicate P-value thresholds: light (P-value < 0.1), medium (P-value < 0.05) and dark (P-value < 0.01).

were stressed by cardiopulmonary exercise testing to evaluate the power of metabolic profiling for discrimination of HF patients vs. healthy controls during and after exercise. We used broad, untargeted screening, and targeted methods for catecholamines and steroids which together allowed for the detection of 252 metabolites.

Figure 3 Impact of exercise (t_1 vs. t_0) on the plasma metabolite profile of HF due to non-ischemic cardiomyopathy (A) and exercise-induced changes in lactate and adrenaline in HF compared with controls (B).
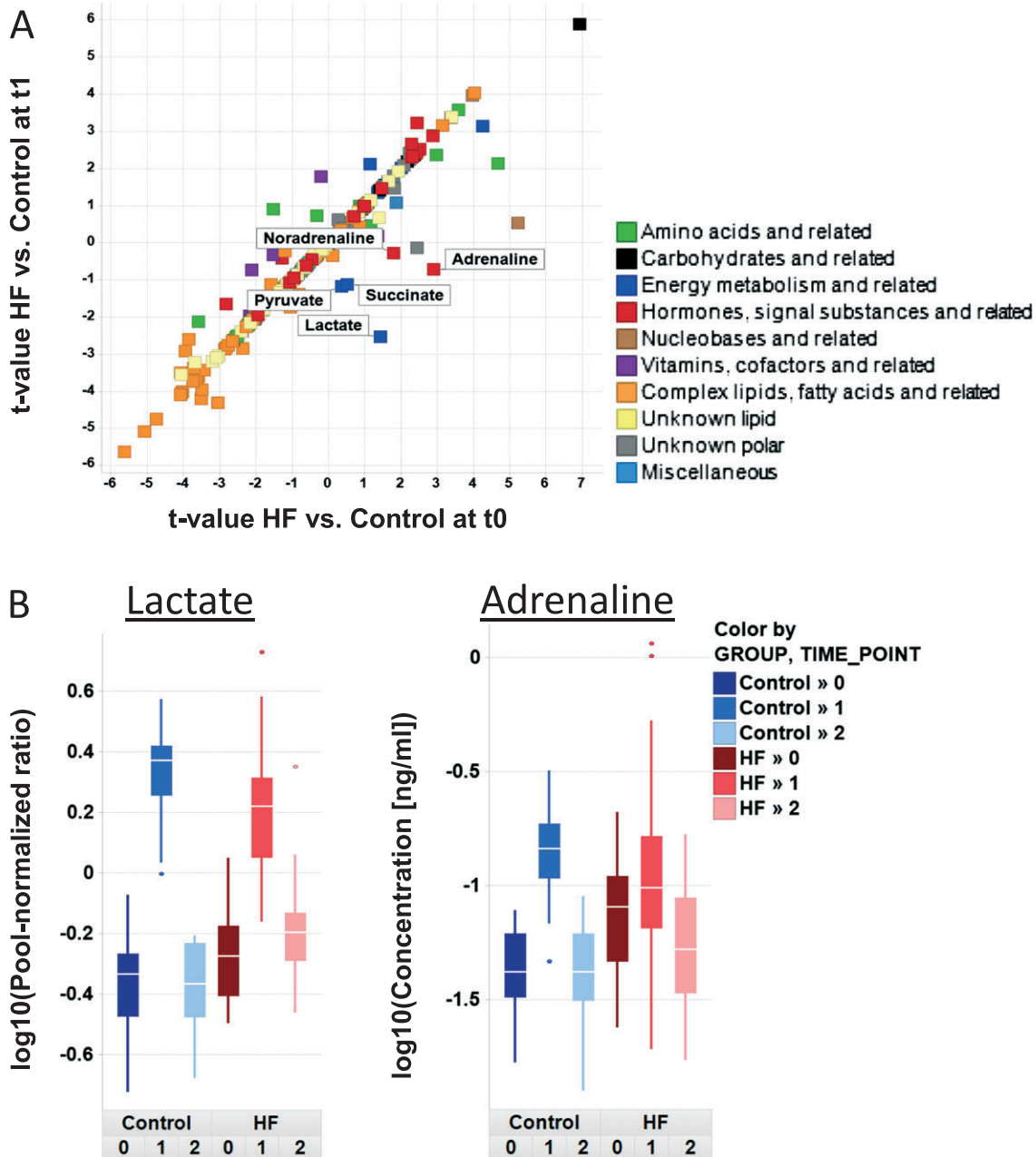
Using these methods, we first identified metabolic changes characteristic of non-ischemic HF patients when compared with healthy controls at rest. Among these, altered levels of amino acids, carbohydrates, catecholamines, and lipids strongly differentiated patients from healthy controls. In particular, we observed decreased levels of complex lipids and fatty acids, notably phosphatidylcholines and sphingolipids. Moreover, reduced glutamine and increased glutamate levels as well as increased levels of adenosine triphosphate (ATP), purine degradation products such as hypoxanthine, uric acid, as well as indices of impaired glucose metabolism were observed in plasma samples of non-ischemic HF patients.

Second, we found that the majority of metabolic differences observed between plasma samples from non-ischemic HF patients and healthy controls at rest persisted throughout

the subsequent sampling time-points during and after exercise. Although exercise testing resulted in a clear and unique pattern of metabolic changes, there were few specific exercise-induced differences in the metabolic profile improving discrimination of non-ischemic HF patients from healthy controls. Notably, HF signatures in NICM patients were stable and largely preserved during and 1 h after stress testing, thereby showing the robustness of the identified metabolic pattern.

Third, the number of significant metabolic differences increased strongly from NYHA I to III and was closely related to decreasing LV-EF. The addition of three metabolites to NYHA functional class furthermore significantly improved prediction of exercise capacity.

The metabolic profile of heart failure at rest and pathway analysis

Our results confirmed data from previous studies on blood metabolites of HF concerning catecholamines, uric acid, and glutamate metabolism[15,16] and added new aspects such as the extensive alterations in lipid metabolism. Notably, several long and very long-chain fatty acids were decreased in the group of HF patients and negatively correlated with disease severity indicated by NYHA class. In particular, we observed significant changes in the levels of phosphatidylcholines and sphingolipids. Very long-chain fatty acids can be predominantly found in phospholipids and consistently our study showed decreased levels of (lyso-)phosphatidylcholines and sphingolipids in the group of HF patients. Sphingolipids are located mainly in the plasma membrane and additionally serve as signalling molecules regulating cellular differentiation, proliferation, and apoptosis (e.g. ceramides).[17] While previous studies revealed myocardial accumulation of triglycerides, fatty acids, and ceramides in the setting of LV dysfunction,[18] others found that levels of phospholipid species commonly containing linoleic acid (C18:2) were significantly reduced in heart tissue of cardiomyopathic hamster model at onset of HF.[19] Our findings of reduced (lyso-)phosphatidylcholines, cholesterol, sphingolipids, free, and lipid-bound fatty acids support the idea that alterations in lipid homeostasis are related to non-ischemic HF, because impaired interaction of membrane-associated protein complexes that modulate cell signalling and myocardial metabolism may be present.[20]

Regarding energy metabolism, prior studies suggested a switch away from fatty acids towards glucose as a preferred substrate of energy generation in the heart in patients with (late-stage) HF with increased glucose and lower fatty acid utilization.[15,21–23] However, the direction and timing of the substrate shift in HF is still unclear.[15,23] Studies in failing human hearts and in animal models of end-stage HF previously demonstrated a substantial down-regulation of both enzymes

of fatty acid oxidation and glucose metabolism, including the GLUT-1, and GLUT-4 glucose transporters.[24] Our findings of significantly decreased fatty acids (sum parameter of free and lipid-bound fatty acids) might support the idea that lipolysis could be reduced because free fatty acids are possible indicators of lipolysis in plasma. A greater dependency on carbohydrate fuels at the expense of fatty acid and acetyl-CoA oxidation in the tricarboxylic acid (TCA) cycle was recently observed in patients with myocardial ischemia by Turer et al.[23] Similar to our findings, Alexander et al. showed increased levels of anaplerotic TCA cycle intermediates including elevations in circulating alpha-ketoglutarate in plasma samples.[15] The TCA cycle plays a predominant part in oxidative phosphorylation as concentrations of TCA cycle intermediates are regimented to allow adequate use of substrates derived from glycolysis and fatty acid oxidation for ATP production.

In addition to these findings, we observed a metabolic profile of disturbed glucose metabolism with some overlap between HF and diabetes metabolites such as elevated glucose, mannose, 2-hydroxybutyrate, and reduced 1,5-anhydrosorbitol. However, after additional correction for diabetes and estimated glomerular filtration rate in univariate analyses, significant differences could still be observed for most metabolites, including glucose, mannose, and 2-hydroxybutyrate but also hypoxanthine, pseudouridine, glutamate, and several lipids. Thus, our data suggest a pathway of disturbed glucose metabolism in non-ischemic HF which may precede manifest diabetes mellitus. These implications are supported by our findings regarding lipid metabolism because reduced plasma levels of LPC (C18:2) and elevated acetylcarnitine have been described to be markers of pre-diabetes.[25] In addition, increased glutamate levels as observed in our study with a high glutamate/glutamine ratio have been found to be related to a higher risk of developing diabetes.[26]

Moreover, our metabolite profiling revealed increased ATP and purine degradation products such as hypoxanthine and uric acid plasma levels indicating an increased catabolism of energy-enriched purines. An increase in uric acid was found to be related to reduced peripheral blood flow and vasodilator function leading to impaired exercise capacity.[27,28] Possible reasons for an up-regulation in HF are alterations in purine metabolism with enhanced ATP turnover and oxidative stress due to xanthine oxidase activity, which generates superoxide anion and hydrogen peroxide.[28]

Metabolic profile in HF during exercise

A subset of the observed changes during exercise is in line with knowledge on exercise physiology. Previous findings in normal subjects suggested exercise-induced elevations in lactate concentrations and products of adenine nucleotide

metabolism in skeletal muscle and plasma that mirror increased anaerobic metabolism and ATP turnover.[10,29] Moreover, an expansion of TCA cycle intermediates may be necessary to augment TCA cycle flux during exercise in order to maintain oxidative metabolism during prolonged exercise.[11,12,30] However, concerning exercise response on plasma concentrations in HF patients, Riley *et al*. previously found that appropriate substrate levels were maintained during exercise and thus concluded that an abnormal metabolic response may not be contributory to exercise intolerance in chronic HF.[31] Similar to our study, only a marginal influence of exercise on the differentiation between HF patients and controls was found. Despite a high number of metabolites significantly correlated with exercise (30.2% at t1/t0), we found a similar number of metabolite changes between HF and controls at t0 and t1 (31.3% vs. 30.7%). Multivariate analysis confirmed these findings demonstrating a similarly predictive group separation between HF patients and controls at all three time points. As a possible explanation for exercise intolerance in HF despite largely maintained metabolites, reduction in total delivery of substrates and skeletal muscle blood flow was previously considered.[31] This explanation is supported by recent studies on impaired oxygen transport and utilization in HF patients.[32] In our study, the slightly 'pre-stressed' metabolic profile with elevated levels of catecholamines and energy metabolites in HF at rest resembles the previous descriptions of the metabolic signature of exercise.[12] In contrast, exercise did not lead to a comparably strong up-regulation of the same metabolites in HF patients and this correlated with reduced exercise performance. Thus, it seems possible that the 'pre-stressed' metabolic/adrenergic phenotype at least partially contributes to exercise intolerance.

Comparing metabolite profiles at baseline and peak exercise revealed that differentiation between HF patients due to NICM and controls did not strongly depend on the sampling time point during the exercise test. Very similar alterations between HF patients and controls at each time point are an unexpected finding as exercise was expected to exaggerate differences. Moreover, the differentiation between non-ischemic HF and controls was not significantly further improved by exercise. Therefore, cardiopulmonary exercise testing may not be required as pre-requisite for a metabolic signature definition of non-ischemic HF. Furthermore, the stability of the metabolic differences between healthy and diseased even under exercise indicates that the identified metabolic changes are quite robust.

Correlation with clinical stages of heart failure

The number of significant metabolic differences increased with higher NYHA stages and decreasing LV-EF. Thus, differences of metabolite levels may be indicative of clinical disease severity and LV systolic function. However, because significantly increased plasma levels of certain metabolites were already observed in asymptomatic HF patients, our results indicate that metabolite disturbances are not limited to higher stages of HF, but begin in the early asymptomatic stage.

Metabolites for prediction of exercise capacity

Determination of $VO_{2,max}$ during symptom-limited cardiopulmonary exercise testing provides an objective assessment of exercise capacity inpatients with HF.[33] Moreover, $VO_{2,max}$ has been found to strongly correlate with cardiac output, mortality risk, and skeletal muscle flow.[34–36] With the limitations of traditional parameters such as NYHA stage for prediction of exercise capacity, additional markers are required to allow a more objective assessment. In our study, the combination of three metabolites with NYHA further improved the predictive power compared with NYHA or other clinical parameters such as LV-EF or NT-proBNP alone. Again, these features comprised metabolites from the lipid classes of phospholipids, which supports our findings that the observed lipid changes at baseline may further contribute to HF progression.

Limitations

The present study was designed as a proof-of-concept study for a metabolic diagnosis of non-ischemic HF and to evaluate whether exercise is necessary to provoke metabolic differences between HF patients and healthy control subjects. Thus, a limitation of our study is the small size of the study population which bears the risk of spectrum bias. Moreover, the results are restricted to male patients with HF due to non-ischemic, idiopathic cardiomoypathy, and cannot be generalized to all HF patients. Because underlying aetiologies of non-ischemic HF may be diverse, patients were only included when the diagnosis of HF due to idiopathic, non-ischemic aetiology was ensured by a standardized routine diagnostic evaluation, including detailed review of clinical history and lab results, 2-D echocardiography and coronary angiography with invasive hemodynamic measurements. In addition, selection criteria aimed to minimize confounding effects of gender, race, and age to provide a study group as homogenous as possible. The longitudinal design of the study by measuring changes after exercise in the same individual further allowed the reduction of external confounders.

Conclusions

Our study identified a distinct metabolic signature in plasma to distinguish male HF patients due to non-ischemic cardiomyopathy from healthy control subjects. Profound alterations

of lipid and amino acid metabolism as well as catecholamines and their catabolites were observed even at rest. Our findings on lipid metabolism in non-ischemic HF with decreased levels of complex lipids, in particular phosphatidylcholines and sphingolipids, support the idea that membrane perturbations may be linked to HF. Furthermore, exercise testing did not significantly improve discrimination between cases and controls. Therefore, cardiopulmonary exercise testing may not be required as part of the diagnostic workflow for a metabolic description of HF patients. The metabolic differences were already seen in asymptomatic HF patients and mirrored disease severity as determined by NYHA functional class and LV systolic function. Finally, combination of metabolites with clinical features further improved the predictive power compared with clinical parameters such as LV-EF or NT-proBNP alone.

Acknowledgements

Conflicts of interest

Drs. Mueller-Hennessen, Sigl, Fuhrmann, Witt, Schmitz, Kastler, Fischer, Müller, Weis, and Katus report grants from the National Genome Research Network (NGFN-transfer) and the German Ministry of Education and Research (BMBF), during the conduct of the study. In addition, Dr. Mueller-Hennessen reports grants from the Medical Faculty of the University of Heidelberg, grants and non-financial support from Metanomics Health GmbH, during the conduct of the study; grants and non-financial support from BRAHMS Thermo Fisher, non-financial support from Daiichi-Sankyo, non-financial support from Bayer Vital, grants and personal fees from Roche Diagnostics, outside the submitted work. Dr. Müller reports grants from Metanomics GmbH, during the conduct of the study. Dr. Giannitsis reports speaker honoraria from Roche Diagnostics, AstraZeneca, Bayer Vital, Daiichi Sankyo, research funding Bayer Vital, consultancy Roche Diagnostics and BRAHMS Thermo Fisher; outside the submitted work. Dr. Weis reports grants and non-financial support from Metanomics Health GmbH, during the conduct of the study. Dr. Katus reports grants from Metanomics Health, during the conduct of the study; grants and personal fees from Roche Diagnostics, grants and personal fees from AstraZeneca, grants and personal fees from Bayer Vital, outside the submitted work. Drs Fuhrmann, Witt, Reszka, Schmitz, Kastler, and Fischer are or were employees of metanomics GmbH (Berlin, Germany) or Metanomics Health GmbH (Berlin, Germany) and are stockholders of BASF SE (Ludwigshafen, Germany). Drs. Sigl, Fuhrmann, Witt, Reszka, Schmitz, Kastler, Fischer, Weis, and Katus have patent applications pending in the scientific area of this study. All other authors declare that they have no conflict of interest with this study.

Funding

Supporting information

Supporting information may be found in the online version of this article.

Table S1. List of metabolites significantly changed in patients vs. controls after additional correction (ANOVA) for diabetes and eGFR.
Table S2. Impact of exercise effects on patients and controls.
Table S3. (A) Correlation analysis of relative VO2max with metabolite levels; (B) No. of metabolites with significant correlation to rel. VO2max according to ontology class; (C) Q2 values for prediction of relative VO2max at T0, T1 and T2 using metabolites.
Figure S1. Effects of exercise on plasma metabolites.
Figure S2. Q2 values of NYHA and addition of metabolites for VO2max prediction at rest (circles) and under exercise (triangles).

References

1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB. Heart disease and stroke statistics-2015 update: a report from the American Heart Association. *Circulation* 2015; **131**: e29–e322.
2. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJ,

Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson LW, Tang WH, Tsai EJ, Wilkoff BL. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol* 2013; **62**: e147–e239.

3. Turer AT. Using metabolomics to assess myocardial metabolism and energetics in heart failure. *J Mol Cell Cardiol* 2013; **55**: 12–18.

4. Nicholson JK, Wilson ID. Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. *Nat Rev Drug Discov* 2003; **2**: 668–676.

5. Allen J, Davey HM, Broadhurst D, Heald JK, Rowland JJ, Oliver SG, Kell DB. High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. *Nat Biotechnol* 2003; **21**: 692–696.

6. Cheng ML, Wang CH, Shiao MS, Liu MH, Huang YY, Huang CY, Mao CT, Lin JF, Ho HY, Yang NI. Metabolic disturbances identified in plasma are associated with outcomes in patients with heart failure: diagnostic and prognostic value of metabolomics. *J Am Coll Cardiol* 2015; **65**: 1509–1520.

7. Stegemann C, Pechlaner R, Willeit P, Langley SR, Mangino M, Mayr U, Menni C, Moayyeri A, Santer P, Rungger G, Spector TD, Willeit J, Kiechl S, Mayr M. Lipidomics profiling and risk of cardiovascular disease in the prospective population-based Bruneck study. *Circulation* 2014; **129**: 1821–1831.

8. Myers J, Prakash M, Froelicher V, Do D, Partington S, Atwood JE. Exercise capacity and mortality among men referred for exercise testing. *N Engl J Med* 2002; **346**: 793–801.

9. Anker SD, Swan JW, Volterrani M, Chua TP, Clark AL, Poole-Wilson PA, Coats AJ. The influence of muscle mass, strength, fatigability and blood flow on exercise capacity in cachectic and non-cachectic patients with chronic heart failure. *Eur Heart J* 1997; **18**: 259–269.

10. Van Hall G, Jensen-Urstad M, Rosdahl H, Holmberg HC, Saltin B, Calbet JA. Leg and arm lactate and substrate kinetics during exercise. *Am J Physiol Endocrinol Metab* 2003; **284**: E193–E205.

11. Gibala MJ, MacLean DA, Graham TE, Saltin B. Tricarboxylic acid cycle intermediate pool size and estimated cycle flux in human muscle during exercise. *Am J Physiol* 1998; **275**: E235–E242.

12. Lewis GD, Farrell L, Wood MJ, Martinovic M, Arany Z, Rowe GC, Souza A, Cheng S, McCabe EL, Yang E, Shi X, Deo R, Roth FP, Asnani A, Rhee EP, Systrom DM, Semigran MJ, Vasan RS, Carr SA, Wang TJ, Sabatine MS, Clish CB, Gerszten RE. Metabolic signatures of exercise in human plasma. *Sci Transl Med* 2010; **2**: 33–37.

13. Jacobs DM, Fuhrmann JC, van Dorsten FA, Rein D, Peters S, van Velzen EJ, Hollebrands B, Draijer R, van Duynhoven J, Garczarek U. Impact of short-term intake of red wine and grape polyphenol extract on the human metabolome. *J Agric Food Chem* 2012; **60**: 3078–3085.

14. Padberg I, Peter E, Gonzalez-Maldonado S, Witt H, Mueller M, Weis T, Bethan B, Liebenberg V, Wiemer J, Katus HA, Rein D, Schatz P. A new metabolomic signature in type-2 diabetes mellitus and its pathophysiology. *PLoS One* 2014; **9**: e85082.

15. Alexander D, Lombardi R, Rodriguez G, Mitchell MM, Marian AJ. Metabolomic distinction and insights into the pathogenesis of human primary dilated cardiomyopathy. *Eur J Clin Invest* 2011; **41**: 527–538.

16. Dunn W, Broadhurst D, Deepak S, Buch M, McDowell G, Spasic I, Ellis D, Brooks N, Kell D, Neyses L. Serum metabolomics reveals many novel metabolic markers of heart failure, including pseudouridine and 2-oxoglutarate. *Metabolomics* 2007; **3**: 413–426.

17. Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol* 2008; **9**: 139–150.

18. Park TS, Hu Y, Noh HL, Drosatos K, Okajima K, Buchanan J, Tuinei J, Homma S, Jiang XC, Abel ED, Goldberg IJ. Ceramide is a cardiotoxin in lipotoxic cardiomyopathy. *J Lipid Res* 2008; **49**: 2101–2112.

19. Maekawa K, Hirayama A, Iwata Y, Tajima Y, Nishimaki-Mogami T, Sugawara S, Ueno N, Abe H, Ishikawa M, Murayama M, Matsuzawa Y, Nakanishi H, Ikeda K, Arita M, Taguchi R, Minamino N, Wakabayashi S, Soga T, Saito Y. Global metabolomic analysis of heart tissue in a hamster model for dilated cardiomyopathy. *J Mol Cell Cardiol* 2013; **59**: 76–85.

20. Jenkins CM, Cedars A, Gross RW. Eicosanoid signalling pathways in the heart. *Cardiovasc Res* 2009; **82**: 240-249.

21. Davila-Roman VG, Vedala G, Herrero P, de las Fuentes L, Rogers JG, Kelly DP, Gropler RJ. Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. *J Am Coll Cardiol* 2002; **40**: 271–277.

22. Tuunanen H, Engblom E, Naum A, Scheinin M, Nagren K, Airaksinen J, Nuutila P, Iozzo P, Ukkonen H, Knuuti J. Decreased myocardial free fatty acid uptake in patients with idiopathic dilated cardiomyopathy: evidence of relationship with insulin resistance and left ventricular dysfunction. *J Card Fail* 2006; **12**: 644–652.

23. Turer AT, Stevens RD, Bain JR, Muehlbauer MJ, van der Westhuizen J, Mathew JP, Schwinn DA, Glower DD, Newgard CB, Podgoreanu MV. Metabolomic profiling reveals distinct patterns of myocardial substrate use in humans with coronary artery disease or left ventricular dysfunction during surgical ischemia/reperfusion. *Circulation* 2009; **119**: 1736–1746.

24. Lei B, Lionetti V, Young ME, Chandler MP, d'Agostino C, Kang E, Altarejos M, Matsuo K, Hintze TH, Stanley WC, Recchia FA. Paradoxical downregulation of the glucose oxidation pathway despite enhanced flux in severe heart failure. *J Mol Cell Cardiol* 2004; **36**: 567–576.

25. Wang-Sattler R, Yu Z, Herder C, Messias AC, Floegel A, He Y, Heim K, Campillos M, Holzapfel C, Thorand B, Grallert H, Xu T, Bader E, Huth C, Mittelstrass K, Doring A, Meisinger C, Gieger C, Prehn C, Roemisch-Margl W, Carstensen M, Xie L, Yamanaka-Okumura H, Xing G, Ceglarek U, Thiery J, Giani G, Lickert H, Lin X, Li Y, Boeing H, Joost HG, de Angelis MH, Rathmann W, Suhre K, Prokisch H, Peters A, Meitinger T, Roden M, Wichmann HE, Pischon T, Adamski J, Illig T. Novel biomarkers for prediabetes identified by metabolomics. *Mol Syst Biol* 2012; **8**: 615.

26. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, Haqq AM, Shah SH, Arlotto M, Slentz CA, Rochon J, Gallup D, Ilkayeva O, Wenner BR, Yancy WS, Jr., Eisenson H, Musante G, Surwit RS, Millington DS, Butler MD, Svetkey LP. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 2009; **9**: 311–326.

27. Bergamini C, Cicoira M, Rossi A, Vassanelli C. Oxidative stress and hyperuricaemia: pathophysiology, clinical relevance, and therapeutic implications in chronic heart failure. *Eur J Heart Fail* 2009; **11**: 444–452.

28. Doehner W, Schoene N, Rauchhaus M, Leyva-Leon F, Pavitt DV, Reaveley DA, Schuler G, Coats AJ, Anker SD, Hambrecht R. Effects of xanthine oxidase inhibition with allopurinol on endothelial function and peripheral blood flow in hyperuricemic patients with chronic heart failure: results from 2 placebo-controlled studies. *Circulation* 2002; **105**: 2619-2624.

29. Hancock CR, Brault JJ, Terjung RL. Protecting the cellular energy state during contractions: role of AMP deaminase. *J Physiol Pharmacol* 2006; **57**: 17-29.

30. Gibala MJ, Gonzalez-Alonso J, Saltin B. Dissociation between muscle tricarboxylic acid cycle pool size and aerobic energy provision during prolonged exercise in humans. *J Physiol* 2002; **545**: 705–713.

31. Riley M, Bell N, Elborn JS, Stanford CF, Buchanan KD, Nicholls DP. Metabolic responses to graded exercise in chronic

heart failure. *Eur Heart J* 1993; **14**: 1484–1488.

32. Poole DC, Hirai DM, Copp SW, Musch TI. Muscle oxygen transport and utilization in heart failure: implications for exercise (in)tolerance. *Am J Physiol Heart Circ Physiol* 2012; **302**: H1050–H1063.

33. Ingle L. Theoretical rationale and practical recommendations for cardiopulmonary exercise testing in patients with chronic heart failure. *Heart Fail Rev* 2007; **12**: 12–22.

34. Arena R, Myers J, Guazzi M. The clinical and research applications of aerobic capacity and ventilatory efficiency in heart failure: an evidence-based review. *Heart Fail Rev* 2008; **13**: 245–269.

35. Reddy HK, Weber KT, Janicki JS, McElroy PA. Hemodynamic, ventilatory and metabolic effects of light isometric exercise in patients with chronic heart failure. *J Am Coll Cardiol* 1988;**12**: 353–358.

36. Cahalin LP, Chase P, Arena R, Myers J, Bensimhon D, Peberdy MA, Ashley E, West E, Forman DE, Pinkstaff S, Lavie CJ, Guazzi M. A meta-analysis of the prognostic significance of cardiopulmonary exercise testing in patients with heart failure. *Heart Fail Rev* 2013; **18**: 79–94.