ORIGINAL RESEARCH

Metabolomic Profiling Identifies Novel Circulating Biomarkers of Mitochondrial Dysfunction Differentially Elevated in Heart Failure With Preserved Versus Reduced Ejection Fraction: Evidence for Shared Metabolic Impairments in Clinical Heart Failure

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***Background*—**Metabolic impairment is an important contributor to heart failure (HF) pathogenesis and progression. Dysregulated metabolic pathways remain poorly characterized in patients with HF and preserved ejection fraction (HFpEF). We sought to determine metabolic abnormalities in HFpEF and identify pathways differentially altered in HFpEF versus HF with reduced ejection fraction (HFrEF).

***Methods and Results*—**We identified HFpEF cases, HFrEF controls, and no-HF controls from the CATHGEN study of sequential patients undergoing cardiac catheterization. HFpEF cases (N=282) were defined by left ventricular ejection fraction (LVEF) ≥45%, diastolic dysfunction grade ≥1, and history of HF; HFrEF controls (N=279) were defined similarly, except for having LVEF <45%. No-HF controls (N=191) had LVEF ≥45%, normal diastolic function, and no HF diagnosis. Targeted mass spectrometry and enzymatic assays were used to quantify 63 metabolites in fasting plasma. Principal components analysis reduced the 63 metabolites to uncorrelated factors, which were compared across groups using ANCOVA. In basic and fully adjusted models, long-chain acylcarnitine factor levels differed significantly across groups (P<0.0001) and were greater in HFrEF than HFpEF (P=0.0004), both of which were greater than no-HF controls. We confirmed these findings in sensitivity analyses using stricter inclusion criteria, alternative LVEF thresholds, and adjustment for insulin resistance.

***Conclusions*—**We identified novel circulating metabolites reflecting impaired or dysregulated fatty acid oxidation that are independently associated with HF and differentially elevated in HFpEF and HFrEF. These results elucidate a specific metabolic pathway in HF and suggest a shared metabolic mechanism in HF along the LVEF spectrum. **(*J Am Heart Assoc*. 2016;5:e003190 doi: 10.1161/JAHA.115.003190)**

Key Words: fatty acid oxidation • heart failure • metabolism • metabolomics • mitochondrial dysfunction

Heart failure (HF) is a complex clinical syndrome affecting 5.7 million people in the United States.[1] Over the past 2 decades, the proportion of patients with HF and preserved ejection fraction (HFpEF) has increased relative to HF with reduced ejection fraction (HFrEF).[2,3] Currently, HFpEF comprises approximately half of the HF population and has morbidity and mortality on par with HFrEF.[4,5] A major public health burden, HFpEF may become the predominant form of HF in the future.[1,2,4]

Despite its epidemiological importance, there are no proven effective therapies for reducing morbidity or mortality in HFpEF; evidence-based interventions are limited to

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Accompanying Datas S1 through S6 and Tables S1 through S10 are available at http://jaha.ahajournals.org/content/5/8/e003190/DC1/embed/inline-supplementary-material-1.pdf

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conventional therapies for symptom relief and comorbidity management.[6] An important obstacle to therapeutic development is poor understanding of HFpEF pathogenesis and pathophysiology.[7–9] Thus, elucidating molecular mechanisms and identifying novel therapeutic targets in HFpEF are critical unmet needs in cardiovascular medicine.[6,10]

Initially, mechanistic investigations in HFpEF centered on myocardial abnormalities, such as diastolic dysfunction and left ventricular hypertrophy, and traditional HF risk factors, such as hypertension and neurohormonal activation. In recent years, greater attention has been focused on systemic mediators, such as oxidative stress, inflammation, and mitochondrial dysfunction.[8,9,11] Emerging evidence is also reviving interest in metabolic impairment as a contributor to HFpEF development and progression.[8,9,12,13] However, these investigations have largely been conducted in animal models; there is a paucity of data characterizing metabolism in human HFpEF.

Metabolomic profiling is an ideal tool for filling this knowledge gap, given that it allows for simultaneous quantification of a wide variety of molecular intermediates from multiple major bioenergetic pathways.[14–16] Thus, in this investigation, we performed targeted, quantitative metabolomic profiling on a cohort of patients with HFpEF and compared profiles to patients with HFrEF and controls without HF. Our primary objective was to determine metabolic abnormalities in HFpEF and identify pathways differentially altered in HFpEF versus HFrEF.

Methods

Study Population

Our study population was selected from the CATHGEN biorepository, which contains clinical data and biological specimens from 9334 patients who underwent cardiac catheterization at Duke University Medical Center (Durham, NC) between January 2001 and December 2010. Details about the CATHGEN biorepository have been previously published.[17,18] After obtaining written informed consent, peripheral blood samples were collected from each patient in EDTA tubes by femoral artery sheath before heparin administration. Samples were immediately cooled to 4°C, centrifuged for 30 minutes to separate plasma, and frozen at −80°C. All patients had been fasting for at least 6 hours before sample collection. As part of the biorepository, targeted quantitative metabolomic profiling has been performed on ≈4000 individuals.

From these CATHGEN subjects, we identified HFpEF cases, HFrEF controls, and controls without HF or diastolic dysfunction (no-HF controls). HFpEF cases were defined by left ventricular ejection fraction (LVEF) ≥45%, diastolic dysfunction grade ≥1, and history of HF.[6,19,20] HFrEF controls were defined by LVEF <45%, diastolic dysfunction grade ≥1, and history of HF. No-HF controls were defined by LVEF ≥45%, normal diastolic function, and no history of HF. Patients were excluded from analysis if they had a history of congenital heart disease, moderate-to-severe valvular disease on echocardiography, cardiac transplantation, or end-stage renal disease.

Given plurality of HFpEF definitions in professional guidelines and in clinical trials,[6,10,19–25] we sought to determine whether results would change significantly with variations of inclusion criteria. Thus, we modified case-control definitions for exploratory sensitivity analyses: Inclusion criteria for the first set of sensitivity cohorts retained all specifications described above, but also required presence or absence of objective HF indicators (elevated n-terminal prohormone of brain natriuretic peptide >400 pg/mL, loop diuretic use, or HF International Classification of Diseases, Ninth Revision code) and excluded all patients with major adverse cardiac events (defined as myocardial infarction consistent with the universal definition,[26] percutaneous coronary intervention, or coronary artery bypass grafting) 1 month before sample collection. Inclusion criteria for the second set of sensitivity cohorts varied only in its LVEF thresholds: HFpEF and no-HF groups had LVEF ≥50% and HFrEF had LVEF <35%. Detailed case-control descriptions are provided in Data S1.

Clinical Phenotyping

Cardiologists providing clinical care at the time of catheterization determined history of HF. Estimates of LVEF were obtained using a ventriculogram at time of cardiac catheterization; if a ventriculogram was not performed, then echocardiogram, nuclear, or cardiac MRI studies within 60 days of sample collection were used. Diastolic function was obtained from echocardiographic assessments made during routine clinical care. To ensure accuracy of previous assessments, a blinded overread of diastolic function in 10% of the study population was performed by cardiologists specializing in echocardiography (S.H.S. amd M.G.K.; Data S2; Table S1).[27] Baseline demographics and clinical characteristics of the study population were collected by the Duke Databank for Cardiovascular Disease using previously described methods.[28] Objective indicators of HF history used for sensitivity analyses were generated during routine clinical care and extracted by automated search of medical records. Insulin resistance (IR) was measured for sensitivity analyses using the Lipoprotein Insulin Resistance Index (LP-IR), a validated IR score having strong associations with glucose disposal rates and homeostasis model assessment of IR in the Multi-Ethnic Study of Atherosclerosis (see Data S3 for additional details).[29]

This research was approved by the Duke University Institutional Review Board.

Laboratory Methods

Tandem flow-injection targeted mass spectrometry (MS/MS) was used to quantify levels of 60 metabolites in each plasma sample: 45 acylcarnitines and 15 amino acids (full metabolite list in Data S4 and Table S2). Absolute quantification of the metabolites was performed using stable isotope dilution as we have done previously.[30,31] Three conventional metabolites (ketones, nonesterified fatty acids, and 3-hydroxybutyrate) were assayed using a Beckman-Coulter DXC600 analyzer and reagents from Wako Chemicals (Richmond, VA). More-detailed methodology and coefficients of variation have been previously reported.[32–36] Metabolite measurements were performed at the Sarah W. Stedman Nutrition Center and Metabolomics Core Laboratory at the Duke Molecular Physiology Institute. IR scores were derived from lipoprotein particle measurements, which were obtained using nuclear magnetic resonance spectroscopy at LipoScience Incorporated (Raleigh, NC).[29,37] Team members were blinded to group status and samples were randomly distributed to mitigate biases.

Statistical Analysis

Metabolites with >25% of values below the lower limit of quantification were excluded from analysis (hexanoyl carnitine, pimelyl-L-carnitine). Three subjects had missing values for ketone metabolites and free fatty acids, which were imputed with full cohort mean values. All metabolites failed formal tests of normality and were log transformed to approximate normal distributions when used in linear regression models.

The 63 plasma metabolites measured for each subject reside in overlapping biological pathways and are thus potentially correlated. To identify metabolite correlations and reduce the burden of multiple comparisons, we performed principal components analysis (PCA) with varimax rotation.[38,39] To aid in factor selection, we used the Kaiser criterion, which retains all factors with eigenvalues greater than 1.0.[40] This reduced the full set of 63 metabolites into 14 uncorrelated factors, which altogether explained 44.6% of the data variance. Individual metabolites with absolute value of factor load ≥0.4 are reported as composing a given factor, as is commonly done based on convention.[30,41–43] To facilitate factor comparisons across groups, general linear models were constructed: "basic" models (adjusted for age, race, and sex) and "fully adjusted" models (adjusted for age, race, sex, body mass index [BMI], diabetes mellitus, hypertension, dyslipidemia, smoking, glomerular filtration rate [GFR], batch, and number of diseased coronary arteries [number of epicardial vessels with ≥75% stenosis graded by 2 observers at the time of catheterization]).

Metabolite factor levels were compared across groups using ANCOVA with Bonferroni-corrected significance of $P<0.0036$ given 14 factor comparisons. Significant metabolite factors were then compared between groups using pair-wise contrasts generated from the fully adjusted ANCOVA. Individual analytes composing significant metabolite factors were compared across groups using ANCOVA; metabolites reaching Bonferroni corrected significance (0.05 divided by the number of metabolite comparisons) were compared between groups using pair-wise contrasts. Significance threshold for all between-group pairwise contrasts was set at $P<0.05$ to limit type 2 error in the setting of Bonferroni-corrected omnibus tests and multiple confirmatory analyses. Unadjusted correlations were evaluated using Spearman's rho. Statistical analyses were performed by D.M.C. and W.G.H. with SAS software (version 9.4; SAS Institute Inc., Cary, NC). All authors had access to the data, take responsibility for its integrity, and have agreed to the manuscript as written.

Results

Baseline Characteristics of the Study Population

Table 1 presents the baseline demographic and clinical characteristics of the study population. When compared to HFrEF controls, HFpEF cases were older (mean age, years: 66±12 vs 61±13), more likely to be women (42% vs 30%), have lower GFR (mean GFR, mL/min per 1.73 m^2: 64±25 vs 66±23), higher BMI (mean BMI, kg/m^2: 31±7 vs 29±8), and more likely to have traditional cardiovascular risk factors, such as hypertension (75% vs 67%), diabetes mellitus (39% vs 37%), and dyslipidemia (60% vs 58%). In contrast, patients with HFrEF were more likely than those with HFpEF to have severe coronary artery disease (CAD; 38% vs 23%) and smoke (51% vs 48%). These characteristics were in alignment with those from major epidemiological studies, thus supporting generalizability of our HFpEF and HFrEF cohorts to broader populations.[44,45]

Plasma levels of the N-terminal prohormone of brain natriuretic peptide (NT-proBNP) were measured as part of routine clinical care for 34% of the overall cohort in the year before sample collection. Median and interquartile (IQR) ranges for each cohort were as follows: HFpEF, 876 pg/mL (IQR, 251–2391); HFrEF, 2641 pg/mL (IQR, 1023–5256); and no-HF, 136 pg/mL (55–378).

Comparison of Metabolite Factor Levels Between HFpEF, HFrEF, and No-HF Patients

Fourteen PCA-derived metabolite factors were identified, clustering in biologically related groupings similar to our previous studies (detailed PCA results in Data S5;

Table 1. Baseline Patient Characteristics

	HFrEF (N=279)	HFpEF (N=282)	No-HF (N=191)	*P* Value*
Age, y	61 (13)	66 (12)	55 (13)	<0.0001
Sex, male	70%	58%	61%	0.02
BMI, kg/m^2	29 (8)	31 (7)	30 (7)	0.007
Race				
Caucasian	66%	70%	80%	0.06
African American	30%	27%	17%	
Hispanic	3%	1%	1%	
Other	1%	2%	2%	
LVEF, %	28 (9)	58 (8)	58 (7)	<0.0001
GFR, mL/min per 1.73 m^2	66 (23)	64 (25)	84 (23)	<0.0001
Hypertension	67%	75%	56%	0.001
Diabetes mellitus	37%	39%	18%	<0.0001
Diseased coronary vessels†				
0	33%	43%	45%	<0.0001
1	16%	19%	22%	
2	14%	15%	17%	
3	38%	23%	16%	
Dyslipidemia	58%	60%	52%	0.32
Smoking	51%	48%	41%	0.26

Values are % or mean±SD. BMI indicates body mass index; GFR, estimated glomerular filtration rate; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LVEF, left ventricular ejection fraction.
**P* values calculated using chi-square tests or Kruskal–Wallis tests, as appropriate.
†Coronary vessel diseased if stenosed >75% when visualized on coronary angiography.

Table S3).[30,46] In the basic model, omnibus ANCOVA identified 2 metabolite factors significant at the Bonferroni-corrected significance threshold (P<0.0036); both were composed of acylcarnitines of varying chain length (Table 2). In the fully adjusted model, the long-chain acylcarnitine (LCAC) factor remained statistically significant (P<0.0001) at the Bonferroni-corrected significance threshold. Mean levels of the LCAC factor were significantly greater in HFrEF controls than HFpEF cases (least square means±SD: 0.504±0.161 vs 0.131±0.162; P=0.0004). Both HFrEF and HFpEF LCAC factor levels were significantly greater than no-HF controls (−0.245±0.173; P<0.0001 and P=0.003 for comparisons with HFrEF and HFpEF, respectively).

Confirmation of LCAC Factor Findings by Analysis of Individual Metabolites

The PCA-derived LCAC factor was primarily composed of 6 individual LCAC metabolites (Table 3), which all differed significantly among the 3 groups in omnibus ANCOVA analyses (all P≤0.0001), corroborating the overall PCA factor results. Plasma concentrations of all 6 metabolites were greatest in HFrEF, intermediate in HFpEF, and lowest in no-HF controls. Five of 6 LCAC metabolite concentrations differed significantly between HFpEF and HFrEF; 4 of 6 differed significantly between HFpEF and no-HF.

Sensitivity Analyses of LCAC Factor

The significant difference observed across groups in LCAC factor levels was confirmed in 3 sensitivity analyses: (1) more strictly defined cohorts using additional objective inclusion criteria to confirm presence or absence of clinical HF; (2) alternate LVEF inclusion criteria with HFpEF and no-HF groups having LVEF ≥50% and HFrEF having LVEF <35%; and (3) adjustment for IR as an additional or surrogate covariate (replacing "history of diabetes mellitus"). In all sensitivity analyses, LCAC factor levels were highest in HFrEF, intermediate in HFpEF, and lowest in no-HF (Datas S1 and S3; Tables S4 through S9), again corroborating the overall results. When compared to the primary analysis, the alternative LVEF threshold and IR sensitivity analyses showed greater HFpEF-HFrEF differences (all P≤0.0002). Differences between HFpEF and no-HF LCAC factor levels were similar in all analyses (P=0.003–0.004).

To provide further confirmation and insight into LCAC factor findings, we combined the primary HFpEF and HFrEF groups into a single HF cohort and stratified patients into 5 groups by LVEF (≥50%, 40–49%. 30–39%, 20–29%, and <20%). We observed a significant linear trend of increasing LCAC levels by decreasing LVEF that persisted after adjustment for 10 clinical covariables (group factor means in fully adjusted model by descending LVEF: 0.20, 0.07, 0.41, 0.57, and 0.83, respectively; trend, P<0.0001).

Discussion

Using targeted, quantitative metabolomic profiling in a large cohort of HFpEF cases with relevant HFrEF and no-HF controls, we successfully identified a group of circulating metabolites that were significantly elevated in HFpEF and HFrEF when compared to no-HF controls, and which discriminate HFpEF from HFrEF. These metabolites, LCACs, were significantly higher in HFrEF than HFpEF, increasing linearly with declining LVEF.

This represents the largest study of circulating metabolites in HFpEF to date. Reflecting impaired or dysregulated fatty acid oxidation, the LCAC elevations observed in this investigation implicate a specific metabolic pathway in HF, identify biomarkers revealing extent of myocardial impairment beyond LVEF, and also suggest a shared metabolic mechanism in HF regardless of LVEF.

Table 2. Metabolite Factor Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls

Factor	Description	ANCOVA*		Pair-wise Comparisons†			Metabolite Factor Mean Values‡		
		Basic§	Fully Adjusted‖	HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=273)	HFpEF (N=263)	No-HF (N=180)
1	Medium-chain acylcarnitines	0.04	0.13						
2	Long-chain dicarboxyl-acylcarnitines	0.008	0.04	0.05	0.30	1.00	0.339 (0.176)	0.084 (0.178)	0.136 (0.190)
3	Short-chain dicarboxyl-acylcarnitines	0.005	0.07						
4	Long-chain acylcarnitines	<0.0001	<0.0001	0.0004	<0.0001	0.003	0.504 (0.161)	0.131 (0.162)	−0.245 (0.173)
5	Ketones and related metabolites	0.13	0.15						
6	C8–C10 acylcarnitines	0.0001	0.09						
7	BCAA and related metabolites	0.04	0.005	0.03	0.01	1.00	0.041 (0.148)	−0.187 (0.150)	−0.254 (0.160)
8	Various amino acids	0.14	0.07						
9	Short-chain acylcarnitines	0.13	0.95						
10	Asparagine, aspartate, 3-hydroxyisovaleryl/malonyl carnitine	0.17	0.11						
11	Histidine, arginine, tigylcarnitine, 3-hydroxylinoleyl/hexadecadienedioyl carnitine	0.11	0.01	1.00	0.001	0.05	−0.352 (0.111)	−0.320 (0.112)	−0.132 (0.119)
12	Valine, glutamine, glutamate	0.008	0.004	0.03	0.008	1.00	−0.447 (0.151)	−0.217 (0.152)	−0.134 (0.162)
13	Alanine, proline, free fatty acids	0.02	0.03	0.40	0.02	0.60	0.125 (0.140)	−0.006 (0.141)	−0.133 (0.151)
14	Docosanoyl-carnitine	0.004	0.03	1.00	0.03	0.20	0.003 (0.135)	0.68 (0.137)	0.247 (0.145)

BCAA indicates branched-chain amino acids; C, carbon chain length; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction.

*Statistical significance in omnibus ANCOVA analyses was $P<0.0036$, reflecting Bonferroni correction for 14 factor comparisons.

†Pair-wise comparisons for factors significant at Bonferroni corrected threshold test for significant between-group differences. *P* values for factors significant at nominal threshold of $P<0.05$ are reported for exploratory purposes. *P* values reflect between-group pair-wise contrasts generated from the fully adjusted ANCOVA procedure.

‡Values are least square means, adjusted for all 11 covariates. SEM is provided beneath each value.

§*P* values for basic model, adjusted for age, race, and sex.

‖*P* values for full model, adjusted for age, race, sex, body mass index, number of diseased coronary arteries, history of diabetes mellitus, hypertension, dyslipidemia, smoking, glomerular filtration rate, and batch.

Cardiac and peripheral organ metabolic impairment have been long recognized as an intrinsic component of HF pathophysiology.[47,48] Although metabolic derangements have been well described in HFrEF patients and animal models, limited data exist characterizing metabolic impairments in HFpEF.[8,9,13] To our knowledge, only 1 other study has investigated circulating metabolites in patients with HFpEF: Zordoky et al. compared plasma metabolite profiles of 24 patients with HFpEF to 20 with HFrEF and 38 without HF. They identified metabolite profiles providing incremental capacity for distinguishing HFpEF from HFrEF and no-HF controls over natriuretic peptides alone.[49] Despite small sample sizes, their results suggest the existence of circulating metabolite abnormalities in HFpEF that differ in severity from HFrEF. We now confirm this by demonstrating significant and differential elevations of plasma LCAC in HFpEF and HFrEF using cohorts 10-fold larger in size and additionally controlling for many confounders, such as CAD angiographic phenotype, IR, renal function, and BMI.

Although novel in the setting of HFpEF, elevated plasma LCACs have been found in previous studies of human HFrEF: Cheng et al. demonstrated significant elevations in plasma LCAC in Ammerican College of Cardiology/American Heart Association Stage C HF patients compared to normal controls.[50] Interestingly, they also found that Stage A HF patients had plasma LCAC levels higher than normal controls, but lower than Stage C HF patients. Similarly, our group recently found that patients with end-stage HF had significantly higher plasma LCAC levels than those with chronic, stable HFrEF.[51] Furthermore, we demonstrated that greater

Table 3. Individual Metabolite Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls

Metabolites		ANCOVA		Pair-wise Comparisons*			Mean Concentration in µmol/L†		
Structure	Trivial Name	Basic‡	Fully Adjusted§	HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=273)	HFpEF (N=263)	No-HF (N=180)
C16	Palmitoyl-carnitine	<0.0001	<0.0001	0.001	<0.0001	0.0007	0.105 (0.04)	0.097 (0.03)	0.084 (0.03)
C18:2	Linoleyl-carnitine	<0.0001	<0.0001	<0.0001	<0.0001	0.04	0.099 (0.05)	0.084 (0.04)	0.072 (0.03)
C18:1	Oleyl-carnitine	<0.0001	<0.0001	<0.0001	<0.0001	0.01	0.185 (0.08)	0.161 (0.07)	0.137 (0.05)
C18	Stearoyl-carnitine	<0.0001	<0.0001	0.09	<0.0001	0.007	0.050 (0.02)	0.047 (0.02)	0.041 (0.02)
C16:1-OH/ C14:1-DC	3-hydroxy-palmitoleoyl-carnitine or cis-5-tetradecenedioyl-carnitine	<0.0001	<0.0001	0.002	<0.0001	0.21	0.011 (0.01)	0.010 (0.005)	0.009 (0.004)
C20:4	Arachidinoyl-carnitine	<0.0001	<0.0001	0.0007	<0.0001	0.49	0.010 (0.01)	0.008 (0.01)	0.007 (0.004)

C indicates carbon chain length; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction.
**P* values reflect between-group pair-wise contrasts generated from the fully adjusted ANCOVA.
†Values are unadjusted mean concentrations. SD is provided beneath each value.
‡*P* values for basic model, adjusted for age, race, and sex.
§*P* values for full model, adjusted for age, race, sex, body mass index, number of diseased coronary arteries, and history of diabetes mellitus, hypertension, dyslipidemia, smoking, glomerular filtration rate, and batch.

plasma LCAC levels were independently associated with worse functional status and mortality in chronic stable HFrEF.[51] Altogether, the plasma LCAC elevations in HFpEF and HFrEF observed in our study and previous investigations may highlight a shared metabolic impairment characteristic of the HF state. Moreover, plasma LCAC concentrations actually may be indicative of the degree of left ventricular dysfunction or HF decompensation; whether they track longitudinally with changes in cardiac function will require other studies.

Biochemically, LCACs are intermediates in the fatty acid ß-oxidation pathway.[52] Structurally, they are long-chain fatty acids (LCFA) esterified to carnitine. Functionally, they facilitate transfer of LCFAs into the mitochondria for ß-oxidation.[52] Although typically short-lived, LCAC accumulate in states of inefficient fatty acid oxidation (FAO), which may be attributed to (1) defects in mitochondrial FAO enzymes or (2) increased FAO relative to tricarboxylic acid (TCA) flux; this leads to a bottleneck of carbon substrates at the TCA cycle.[53,54] Such defects can be caused or exacerbated by IR, which has, in turn, been associated with elevations in plasma LCAC.[54–57] However, we found no correlation between plasma LCAC and IR in this study; this suggests that the observed LCAC elevations were not driven by IR.[54–57] Regardless of the precise cause, LCAC are transported out to the plasma, where they are subsequently metabolized in several tissues (especially skeletal muscle, liver, and heart) or excreted in urine or bile.[52,56,57] The relative contribution of individual organs to the plasma LCAC pool has not been well characterized in humans; however, animal and cell culture studies suggest that plasma LCAC levels predominantly reflect liver and skeletal muscle secretion as well as renal excretion.[56–58]

Despite incomplete understanding of their sources, LCAC are circulating biomarkers reflecting several pathophysiological processes relevant to HF.[13] When elevated, plasma LCAC reflect underlying mitochondrial dysfunction and dysregulated carbohydrate/fatty acid metabolism, which have both been recognized as viable therapeutic targets in HF.[52,56,59,60] Similarly, elevated plasma LCAC has been used for decades as a screening measure for genetic deficiencies of FAO enzymes.[59] Furthermore, elevated plasma LCACs are associated with impaired physical performance in geriatric populations[61] and also shown to be predictive of poorer physical function, worsened anemia, and increased cardiovascular mortality in hemodialysis-dependent patients.[62]

From a mechanistic standpoint, mounting evidence suggests that plasma LCACs have direct, adverse cellular and physiological effects.[63–68] First, by activating cyclooxygenase-2 signaling and increasing interleukin-6 release, LCACs may promote local skeletal muscle inflammation and systemic inflammation, which are both pathophysiological targets in HF.[63–65,69] Second, LCACs stimulate reactive oxygen species production and promote cellular stress through activation of c-Jun amino-terminal kinase, p38 mitogen-activated protein kinase, and the apoptotic caspase-3 protein.[56,63] Third, LCACs may promote malignant arrhythmias by (1) modulating deactivation kinetics of voltage-gated potassium channels and (2) broadly increasing intracellular calcium concentrations by promoting net calcium efflux from the sarcoplasmic reticulum.[66–68] Last, LCACs may not only reflect, but also exacerbate IR by inducing serine phosphorylation of insulin receptor substrate 1.[56] Through these actions and potential downstream effects on the cyclic guanosine monophosphate/protein kinase

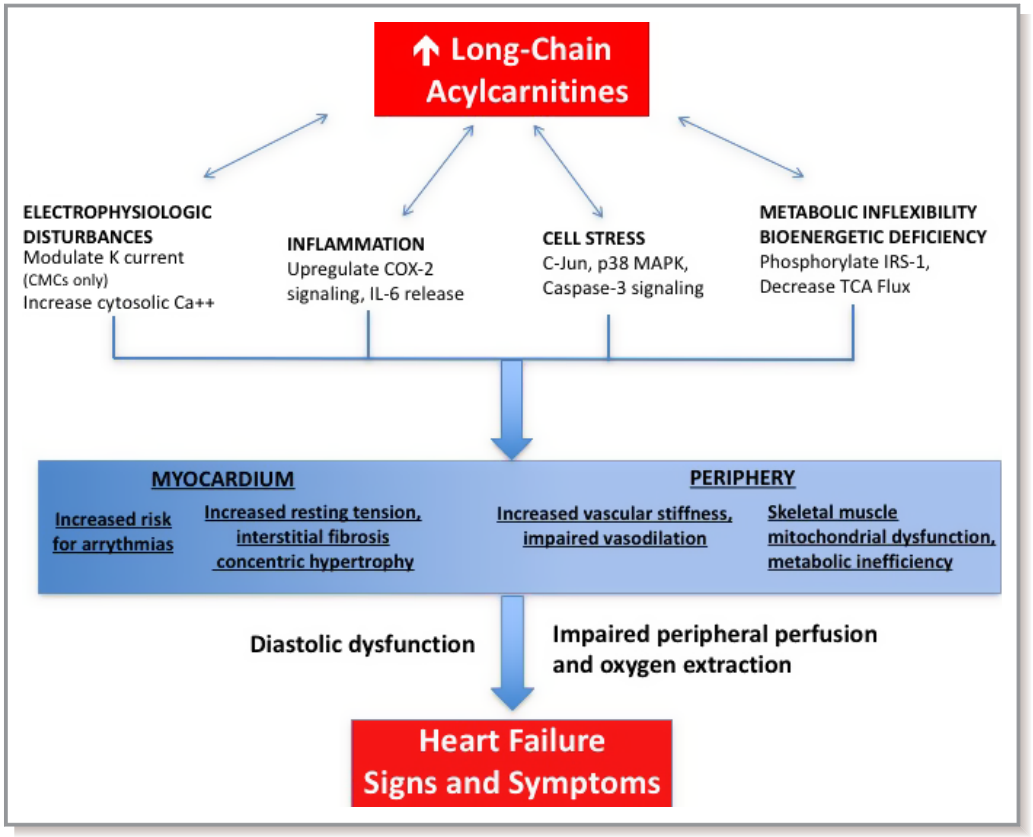

Figure. Proposed model for plasma long-chain acylcarnitine contributions to the heart failure phenotype. Long-chain acylcarnitines accumulate in tissues and plasma in states of inefficient ß-oxidation.[59] Such accumulation causes electrophysiological disturbances, cell stress, and release of circulating inflammatory mediators.[63–68] These may, in turn, activate the cyclic guanosine monophosphate/protein kinase G pathway, previously implicated in the genesis of ventricular fibrosis and hypertrophy as well as vascular stiffness and impaired vasodilation.[9,56] Through these mechanisms, long-chain acylcarnitines may contribute to the heart failure phenotype. Ca^{++} indicates calcium; CMC, cardiomyocyte; COX, cyclooxygenase; IL, interleukin; IRS, insulin receptor substrate; K, potassium; MAPK, mitogen-associated protein kinase; TCA, tricarboxylic acid.

G pathway, elevated LCACs may reflect, promote, or exacerbate myocardial and peripheral pathologies contributing to the HF phenotype (Figure).[9]

Study Strengths and Limitations

The findings observed in this study were robust, observed using broad cohort inclusion criteria mirroring day-to-day clinical practice[6] and confirmed in 3 sensitivity analyses featuring (1) stricter inclusion criteria resembling clinical trials[20]; (2) alternate LVEF inclusion criteria thresholds; and (3) adjustment for IR as an additional or surrogate covariate (in lieu of diabetes mellitus). In addition, we studied 3 major groups along the HF spectrum; used large cohort sizes compared with previous HF metabolomic analyses; used a targeted metabolomic approach permitting metabolite quantitation; and used state-of-the-art assessment and analytic techniques.[18,30–32]

Nevertheless, there were several limitations. First, this study lacked replication cohorts; however, plasma levels of individual LCAC metabolites in our HFpEF and no-HF patients were similar to those in corresponding external cohorts from the RELAX trial and the CATHGEN biorepository at large (Data S6; Table S10), thereby supporting generalizability of our results to broader populations. Nevertheless, our findings should be validated in other, well-defined HFpEF, HFrEF, and no-HF cohorts. Second, the study population was comprised of patients undergoing cardiac catheterization, which could bias our findings by overrepresentation of ischemic phenotypes. We suggest that such bias is unlikely for the following 2 reasons: (1) Results were adjusted for an angiographically determined number of diseased coronary vessels as well as 9 other demographic and clinical risk factors; (2) and the prevalence of CAD in our HF cohorts (57% in HFpEF, 67% in HFrEF) was in alignment with epidemiological estimates.[70–72]

The third limitation of this study was that clinical data were obtained from routine care and thus may have suboptimal accuracy. For this reason, we assessed the accuracy of diastolic dysfunction class with a 10% blinded over read by experienced echocardiographers. We found a good concordance between past and present assessments, which justified our use of the routine clinical measurements (Data S2). We did not evaluate the accuracy of all clinical variables (eg, LVEF, number of diseased coronary vessels, clinical history of HF, GFR, or diabetes mellitus); we rather relied on clinical assessments made by Duke cardiologists and the rigorous data collection practices of the Duke Databank for Cardiovascular Disease, which has been used in many previous studies.

Fourth, the use of targeted metabolite profiling allowed for absolute quantitation of metabolite concentrations, but limited the breadth of analysis; we measured 45 acylcarnitines and 15 amino acids in this study. Although these metabolites represent a small portion of the human metabolome in terms of absolute numbers, they report on pathways critical for cellular and organism-level homeostasis: fatty acid, carbohydrate, amino acid, and urea metabolism. These particular metabolites were chosen for several reasons: (1) An expanding body of evidence has implicated impairments in fatty acid and carbohydrate oxidation in HF pathophysiology, which could be reflected in acylcarnitine elevations[13,50,73–77]; (2) previous metabolomics investigations have identified derangements in plasma levels of these metabolites in HF patients[49,50,78–80]; and (3) acylcarnitines and their derivatives have been suggested to have intrinsic physiological effects that could contribute to the HF phenotype.[66–68,81,82]

Fifth, our study highlights potentially provocative associations, but does not prove a causal role for elevated LCAC in the generation or exacerbation of HF phenotypes. Last, this investigation was agnostic to tissue source of circulating metabolites; obtaining more insight as to the mechanisms of these findings will be important for understanding the role of LCAC and FAO deficiencies in HF pathophysiology.

Conclusions

In the largest metabolomic investigation of HFpEF to date, we identified a signature of circulating metabolites significantly elevated in patients with HFpEF and HFrEF compared to no-HF controls. Importantly, these LCAC metabolites were significantly higher in HFrEF than HFpEF, inversely related to LVEF. As a reflection of impaired or dysregulated FAO, the elevated plasma LCAC observed may suggest a shared metabolic impairment of the HF clinical syndrome independent of LVEF.[13,53,54] Given that FAO impairments or dysregulation may result from a variety of mitochondrial insults, further investigation will be needed to identify the causal processes underlying the LCAC elevations reported in this study.[12,83] Given mounting evidence that LCACs are proinflammatory, arrhythmogenic, and induce cell stress, our findings may suggest a benefit for mitochondrial therapies that decrease LCAC production by increasing glucose oxidation, decreasing FAO flux, or improving mitochondrial function by antioxidant activity.[63–68] Future studies should assess the value of LCAC levels in risk-stratifying HFpEF and HFrEF populations and further investigate the mechanisms by which they reflect, generate, and/or exacerbate HF pathophysiology.

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SUPPLEMENTAL MATERIAL

Data S1. Sensitivity analyses to determine impact of alternate cohort definitions

We sought to determine whether results would change significantly with implementation of stricter definitions of HFpEF, HFrEF, and no-HF controls. Accordingly, in this sensitivity analysis, we defined our cohorts as described below. Results are shown in Supplemental Tables S4 and S5.

HFpEF cases were defined by left ventricular ejection fraction (LVEF) > 45%, diastolic dysfunction grade ≥ 1, clinical history of HF determined by cardiologists at the time of catheterization, and one of the following objective indicators of HF in the 12 months before sample collection: elevated NT-proBNP (>400 pg/mL), loop diuretic use, or HF ICD-9 code associated with a clinical encounter. HFrEF controls were defined similarly to HFpEF cases, with the exception of having LVEF < 45%. No-HF controls were defined by LVEF > 45%, normal diastolic function, absence of heart failure symptoms, and no elevated NT-proBNP (>400 pg/mL), loop diuretic use, or HF ICD-9 code EVER before sample collection. Additionally, all patients were excluded who had a major adverse cardiac event (myocardial infarction, coronary artery bypass grafting, percutaneous coronary intervention) within 1 month of catheterization. Objective indicators of HF history were generated during routine clinical care and extracted by automated search of medical records.

We also sought to determine whether using alternate LVEF thresholds would impact results of our analyses. Accordingly, we regenerated our cohorts using all of the same inclusion criteria as the primary cohorts except that the HFpEF and No-HF groups had LVEF ≥ 50% and HFrEF LVEF < 35%.

Data S2. Approach to diastolic dysfunction classification

Diastolic function assessments were made during routine clinical care. Given temporal variation in diastolic function classification practices, a 10% overread was performed by experienced echocardiographers (S.H.S. and M.G.K.) to ensure accuracy of these assessments. Diastolic classifications made during overreading were based on American Society of

Echocardiography guidelines (Supplemental Table S1, below). Concordance between present overreading and prior assessments was 84%, which was deemed to be an acceptable level of agreement to support using previous clinical assessments. [1]

Data S3. Sensitivity analyses to determine the impact of insulin resistance

As noted in the Discussion, elevations in plasma LCAC may be a cause or consequence of insulin resistance (IR). Although we reported and adjusted for overt diabetes in our analyses, it is possible that IR exerts an incremental mediation effect. To determine the impact of IR on the relationships observed between HFpEF, HFrEF, no-HF, and plasma LCAC, we performed several sensitivity analyses. In addition to repeating the primary analysis with adjustment for IR, we assessed correlations between IR and LCACs directly.

We used the Lipoprotein Insulin Resistance Index (LP-IR), a validated IR measurement derived from nuclear magnetic resonance (NMR)-based lipoprotein subclass particle size and concentration.[2] The LP-IR index has been shown to have strong correlations with glucose disposal rate (GDR) and HOMA-IR. [2]

Correlations between LCAC and IR

To determine the relationship between LCAC and IR directly, we evaluated unadjusted correlations between Factor 4 (LCAC) and LP-IR for the full cohort using Spearman's rho. We found no correlation between LCAC and LP-IR (r =-0.04; P = 0.3).

Impact of IR Adjustment on Primary Analysis Results

To determine whether IR mediates the relationship between HFpEF, HFrEF, no-HF, and plasma LCAC levels, we created two separate general linear models. The first model included all of the covariates used in the primary analysis (age, race, sex, body mass index, number of diseased coronary arteries, history of diabetes, hypertension, dyslipidemia, smoking, glomerular filtration rate, batch), and added LP-IR. The second model included all of the covariates used in the primary

analysis, but replaced 'history of diabetes' with LP-IR levels. We performed multivariate adjusted analysis of covariance (ANCOVA) with post-hoc pairwise comparisons using both models.

As shown in Supplemental Tables S6 and S7, this adjustment did not change the results. Specifically, LCAC factor levels remained significantly different among groups in the omnibus ANCOVA for both IR sensitivity analyses (both $P<0.0001$). Similarly, all pairwise comparisons of LCAC factor levels remained significantly different in the IR sensitivity analyses. Additionally, the trends in mean LCAC factor concentrations were preserved in the IR sensitivity analyses, with LCAC levels highest in HFrEF, intermediate in HFpEF, and lowest in no-HF patients. Analyses of individual LCAC metabolites in HFpEF, HFrEF, and no-HF patients (Supplemental Tables S8 and S9) confirmed findings from the IR sensitivity analyses and were concordant with those from the primary analysis. Altogether, these results suggest that LCAC factor findings were not driven by IR.

Data S4: Complete list of measured metabolites

See Supplemental Table S2 below for the complete list of metabolites measured in this investigation.

Data S5. Detailed results of principal components analysis

Principal components analysis reduced the full set of 63 metabolites into a smaller number of uncorrelated factors. Fourteen factors exceeded the Eigenvalue threshold of 1.0, and are listed in Supplemental Table S3 below. This threshold is based on the Kaiser Criterion, which allows parsimonious selection of factors explaining a significant amount of inter-subject variation. Component metabolites are listed in order of magnitude of factor load, with only those having a factor load $\geq |0.4|$ listed. Variance refers to the proportion of overall variance explained by a given factor.

Data S6: Plasma LCAC means for additional HFpEF, HFrEF, and control cohorts

To provide insight into how the plasma LCAC values of our cohorts compare with those reported in similar populations, we have provided baseline plasma LCAC means for three additional cohorts: 1) N=161 patients enrolled in the RELAX trial of sildenafil in HFpEF; [3] 2) N=453 patients enrolled in the HF-ACTION trial of exercise in HFrEF; [4] and 3) N=3653 patients without HF enrolled in CATHGEN who were not included in the primary analysis. [5]

As shown in Table S10 below, we found similar levels of individual LCAC metabolites for HFpEF and no-HF controls between the respective cohorts. For HFrEF, there were some metabolites that were higher in CATHGEN as compared with HF-ACTION, likely related to the fact that HF-ACTION participants were outpatients and CATHGEN participants included inpatients with more acute heart failure presentations. Results of these analyses support generalizability of the present findings to broader populations.

TABLE S1: Parameters Used in Diastolic Dysfunction Class Overreading

Grade	Mitral E/A ratio	E/E' ratio	Left Atrial (LA) Size (ml/m^2)	Deceleration Time (ms)	Pulmonary Vein Flow
0 (none)	>0.8	<8	< 34	> 200	D>S
I (mild)	<0.8	<8	≥ 34	> 200	S>D
II (moderate)	≥1	>10	≥ 34	160-200	S>D
III/IV (severe)	≥2	≥13	≥ 34	< 160	S>D

TABLE S2: List of Measured Metabolites	
Short name*	**Trivial names**
C2	Acetyl carnitine
C3	Propionyl carnitine
C4/Ci4	Butyryl carnitine or isobutyryl carnitine
C5:1	Tiglyl carnitine or 3-methyl crotonyl carnitine
C5	Isovaleryl, 3-methylbutyryl carnitine , 2-Methylbutyryl, valeryl or pivaloyl carnitine
C4-OH	D-3-Hydroxy-butyryl carnitine, L-3-hydroxybutyryl carnitine
C6	Hexanoyl carnitine
C5-OH/C3-DC	3-Hydroxy-isovaleryl carnitine or malonyl carnitine
Ci4-DC/C4-DC	Methylmalonyl carnitine or succinyl carnitine
C8:1	Octenoyl carnitine†
C8	Octanoyl carnitine
C5-DC	Glutaryl carnitine, ethylmalonyl carnitine
C8:1-OH/C6:1-DC	3-Hydroxy- octenoyl carnitine or hexenedioyl carnitine
C8-OH/C6-DC	3-hydroxy octanoyl carnitine or adipoyl carnitine, 3-methylglutaryl carnitine
C10:3	Decatrienoyl carnitine†
C10:2	Decadienoyl carnitine†
C10:1	Decenoyl carnitine†
C10	Decanoyl carnitine
C7-DC	Pimeloyl carnitine, heptanedioyl carnitine
C10:1-OH/C8:1-DC	3-Hydroxy-decenoyl carnitine or octadecenedioyl carnitine
C10-OH/C8-DC	3-Hydroxy-decanoyl carnitine or suberoyl carnitine
C12:1	Dodecenoyl carnitine†
C12	Lauroyl carnitine
C12-OH/C10-DC	3-Hydroxy-dodecanoyl carnitine or sebacoyl carnitine
C14:2	Tetradecadienoyl carnitine†
C14:1	Tetradecenoyl carnitine†
C14	Myristoyl carnitine
C14:1-OH/C12:1-DC	3-Hydroxy-tetradecenoyl carnitine or dodecenedioyl carnitine
C14-OH/C12-DC	3-Hydroxy-tetradecanoyl carnitine or dodecanedioyl carnitine
C16:2	Hexadecadienoyl carnitine†
C16:1	Palmitoleoyl carnitine†
C16	Palmitoyl carnitine

C16:1-OH/C14:1-DC	3-Hydroxy-palmitoleoyl carnitine or cis-5-tetradecenedioyl carnitine
C16-OH/C14-DC	3-Hydroxy-hexadecanoyl carnitine or tetradecanedioyl carnitine
C18:2	Linoleyl carnitine
C18:1	Oleyl carnitine
C18	Stearoyl carnitine
C18:2-OH/C16:2-DC	3-Hydroxy-linoleyl carnitine or hexadecadienedioyl carnitine
C18:1-OH/C16:1-DC	3-Hydroxy-octadecenoyl carnitine or hexadecanedioyl carnitine
C18-OH/C16-DC	3-Hydroxy-octadecanoyl carnitine or hexadecanedioyl carnitine, thapsoyl carnitine
C20:4	Arachidonoyl carnitine
C20	Arachidoyl carnitine, eicosanoyl carnitine
C18:1-DC	Octadecenedioyl carnitine
C20-OH/C18-DC/C22:6	3-Hydroxy-eicosanoyl carnitine or octadecanedioyl carnitine or docosahexaenoyl carnitine
C22	Docosanoyl carnitine, Behenoyl carnitine
GLY	Glycine
ALA	Alanine
SER	Serine
PRO	Proline
VAL	Valine
LEU/ILE	Leucine/Isoleucine
MET	Methionine
HIS	Histidine
PHE	Phenylalanine
TYR	Tyrosine
ASX	Aspartic acid/asparagine
GLX	Glutamine/glutamate
ORN	Ornithine
CIT	Citrulline
ARG	Arginine
FFA	Total free fatty acids
HBUT	β-Hydroxybutyrate
KET	Ketones

* Some metabolite isomers and isobars could not be differentiated by flow injection tandem mass spectrometry; potential isomers or isobars are listed where applicable. † Positions of double bond(s) uncertain. Abbreviations: C indicates acylcarnitine carbon chain length; OH, hydroxyl; DC, dicarboxyl.

TABLE S3: Peripheral Blood Metabolite Principal Components

Factor	Description	Component Metabolites	Eigenvalue	Variance
1	Medium-chain acylcarnitines	C8, C10, C12, C14:1, C14, C16:2, C16:1, C14:2, C12:1, C10:1	14.06	7.17
2	Long-chain dicarboxyl-acylcarnitines	C20:1-OH/C18:1-DC, C18-OH/C16-DC, C20-OH/C18-DC, C16-OH/C14-DC, C18:1-OH/C16:1-DC, C20, C12-OH/C10-DC, C14-OH/C12-DC	5.64	5.61
3	Short-chain dicarboxyl-acylcarnitines	C5-DC, C6:1-DC/C8:1-OH, C8:1-DC, C6-DC, Ci4-DC/C4-DC, C10-OH/C8-DC, C12-OH/C10-DC, Citrulline	4.86	5.12
4	Long-chain acylcarnitines	C18:1, C18:2, C18, C16, C20:4, C16:1-OH/C14:1-DC	3.80	4.34
5	Ketones and related metabolites	Ketones, ß-hydroxybutyrate, ß-hydroxybutyryl-carnitine, acetylcarnitine, alanine	2.52	4.19
6	C8-C10 acylcarnitines	C10:3, C8:1, C10:2, C10:1	2.47	3.08
7	BCAA and related metabolites	phenylalanine, tyrosine, leucine/isoleucine, valine, methionine,	2.32	2.88
8	Various amino acids	glycine, methionine, serine, ornithine, arginine, C5:1, proline	1.60	2.79
9	Short-chain acylcarnitines	C4/Ci4, C3, C5's	1.47	2.31
10	3-hydroxyisovaleryl / malonyl carnitine, asparagine, aspartate,	C5-OH/C3-DC, asparagine/aspartate,	1.42	1.65
11	Tigylcarnitine, histidine, 3-hydroxy linoleyl /hexadeca-dienedioylcarnitine, arginine	C5:1, histidine, C18:2-OH/C16:2-DC, arginine	1.22	1.49
12	Glutamine, glutamate, valine	glutamine/glutamate, valine	1.12	1.43
13	Alanine, proline, free fatty acids	alanine, proline, circulating free (non-esterified) fatty acids	1.07	1.35
14	Docosanoylcarnitine	C22	1.01	1.16

TABLE S4: Metabolite Factor Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls Using Strict Cohort Definitions

Factor	Description	ANCOVA*		Pairwise Comparisons§			Metabolite Factor Mean Values¶		
		*Basic**	*Fully Adjusted***	HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=136)	HFpEF (N=117)	No-HF (N=129)
1	Medium-chain acylcarnitines	0.04	0.13						
2	Long-chain dicarboxyl-acylcarnitines	0.008	0.04						
3	Short-chain dicarboxyl-acylcarnitines	0.005	0.07						
4	Long-chain acylcarnitines	<0.0001	<0.0001	0.0004	<0.0001	0.003	0.458 (0.219)	0.007 (0.219)	-0.334 (0.221)
5	Ketones and related metabolites	0.13	0.15						
6	C8-C10 acylcarnitines	0.0001	0.09						
7	BCAA and related metabolites	0.04	0.005	0.03	0.01	1.00	0.264 (0.219)	-0.006 (0.219)	-0.213 (0.221)
8	Various amino acids	0.14	0.07						
9	Short-chain acylcarnitines	0.13	0.95						
10	Asparagine, aspartate, 3-hydroxyisovaleryl / malonyl carnitine	0.17	0.11						
11	Histidine, arginine, tigylcarnitine, 3-hydroxylinoleyl / hexadecadienedioyl carnitine	0.11	0.01	1.00	0.01	0.05	-0.395 (0.175)	-0.390 (0.175)	-0.112 (0.176)
12	Valine, glutamine, glutamate	0.008	0.004	0.03	0.008	1.00	-0.694 (0.229)	-0.291 (0.229)	-0.228 (0.231)
13	Alanine, proline, free fatty acids	0.02	0.03	0.36	0.02	0.61	0.067 (0.213)	-0.190 (0.213)	-0.094 (0.215)
14	Docosanoyl-carnitine	0.004	0.03	1.00	0.03	0.18	-0.258 (0.211)	-0.119 (0.211)	0.101 (0.213)

*Statistical significance in omnibus ANCOVA analyses was $P<0.0036$, reflecting Bonferroni correction for 14 factor comparisons. † P values for basic model, adjusted for age, race and sex. ‡ P values for full model, adjusted for age, race, sex, body mass index, number of diseased coronary arteries, history of diabetes, hypertension, dyslipidemia, smoking, glomerular filtration rate, and batch. § Pairwise comparisons for factors significant at Bonferroni corrected threshold test for significant between-group differences. P values for factors significant at nominal threshold of $P<0.05$ are reported for exploratory purposes. P values reflect between-group pairwise contrasts generated from the fully adjusted ANCOVA procedure. ¶ Values are least square means, adjusted for all 11 covariates. Standard error of the mean is provided beneath each value. Abbreviations: HFpEF indicates heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HF, heart failure; ANCOVA, analysis of covariance; BCAA, branched-chain amino acids; C, carbon chain length.

TABLE S5: Metabolite Factor Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls Using Alternate LVEF Thresholds

Factor	Description	ANCOVA*		Pairwise Comparisons§			Metabolite Factor Mean Values¶		
		Basic†	*Fully Adjusted‡*	HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=189)	HFpEF (N=232)	No-HF (N=166)
1	Medium-chain acylcarnitines	0.007	0.04	0.27	0.05	1.00	0.345 (0.142)	0.196 (0.140)	0.109 (0.150)
2	Long-chain dicarboxyl-acylcarnitines	0.003	0.02	0.04	0.09	1.00	0.297 (0.212)	-0.027 (0.208)	-0.016 (0.223)
3	Short-chain dicarboxyl-acylcarnitines	0.009	0.17						
4	Long-chain acylcarnitines	<0.0001	<0.0001	0.0001	<0.0001	0.003	0.611 (0.188)	0.138 (0.185)	-0.277 (0.198)
5	Ketones and related metabolites	0.07	0.16						
6	C8-C10 acylcarnitines	<0.0001	0.02	0.67	0.02	0.25	0.195 (0.146)	0.086 (0.143)	-0.085 (0.154)
7	BCAA and related metabolites	0.01	0.002	0.02	0.003	1.00	0.017 (0.172)	-0.268 (0.169)	-0.376 (0.181)
8	Various amino acids	0.13	0.05	0.05	0.44	1.00	-0.128 (0.148)	0.093 (0.146)	0.018 (0.156)
9	Short-chain acylcarnitines	0.10	0.81						
10	Asparagine, aspartate, 3-hydroxyisovaleryl / malonyl carnitine	0.42	0.23						
11	Histidine, arginine, tigylcarnitine, 3-hydroxylinoleyl / hexadecadienedioyl carnitine	0.34	0.05	1.00	0.05	0.19	-0.286 (0.127)	-0.235 (0.125)	0.076 (0.134)
12	Valine, glutamine, glutamate	0.0007	0.0008	0.01	0.002	1.00	-0.473 (0.168)	-0.172 (0.165)	-0.074 (0.177)
13	Alanine, proline, free fatty acids	0.03	0.02	0.51	0.01	0.27	-0.061 (0.161)	-0.074 (0.158)	-0.257 (0.169)
14	Docosanoyl-carnitine	0.0005	0.004	0.32	0.003	0.19	0.026 (0.153)	0.178 (0.150)	0.369 (0.161)

*Statistical significance in omnibus ANCOVA analyses was $P<0.0036$, reflecting Bonferroni correction for 14 factor comparisons. † *P* values for basic model, adjusted for age, race and sex. ‡ *P* values for full model, adjusted for age, race, sex, body mass index, number of diseased coronary arteries, history of diabetes, hypertension, dyslipidemia, smoking, glomerular filtration rate, and batch. § Pairwise comparisons for factors significant at Bonferroni corrected threshold test for significant between-group differences. *P* values for factors significant at nominal threshold of $P<0.05$ are reported for exploratory purposes. *P* values reflect between-group pairwise contrasts generated from the fully adjusted ANCOVA procedure. ¶ Values are least square means, adjusted for all 11 covariates. Standard error of the mean is provided beneath each value. Abbreviations: HFpEF indicates heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HF, heart failure; ANCOVA, analysis of covariance; BCAA, branched-chain amino acids; C, carbon chain length.

TABLE S6: Adjusted Metabolite Factor Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls, Controlling for History of Diabetes and Insulin Resistance*

Factor	Description	ANCOVA	Pairwise Comparisons‡			Metabolite Factor Mean Values§		
		P-value †	HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=263)	HFpEF (N=273)	No-HF (N=183)
1	Medium-chain acylcarnitines	0.16						
2	Long-chain dicarboxyl-acylcarnitines	0.0009	0.002	0.01	1.00	0.317 (0.136)	0.036 (0.137)	0.041 (0.146)
3	Short-chain dicarboxyl-acylcarnitines	0.13						
4	Long-chain acylcarnitines	<0.0001	0.0002	<0.0001	0.003	0.454 (0.155)	0.070 (0.156)	-0.299 (0.167)
5	Ketones and related metabolites	0.36						
6	C8-C10 acylcarnitines	0.06						
7	BCAA and related metabolites	0.007	0.046	0.01	1.00	0.042 (0.151)	-0.182 (0.152)	-0.259 (0.162)
8	Various amino acids	0.03	0.07	0.10	1.00	-0.150 (0.130)	0.032 (0.131)	0.042 (0.140)
9	Short-chain acylcarnitines	0.90						
10	Asparagine, aspartate, 3-hydroxyisovaleryl / malonyl carnitine	0.15						
11	Histidine, arginine, tigylcarnitine, 3-hydroxylinoleyl / hexadecadienedioyl carnitine	0.01	1.00	0.009	0.09	-0.374 (0.112)	-0.316 (0.112)	-0.143 (0.120)
12	Valine, glutamine, glutamate	0.007	0.16	0.007	0.54	-0.372 (0.147)	-0.199 (0.147)	-0.060 (0.158)
13	Alanine, proline, free fatty acids	0.02	0.40	0.02	0.54	0.131 (0.138)	0.005 (0.138)	-0.126 (0.148)
14	Docosanoyl-carnitine	0.06						

* Statistical significance in omnibus ANCOVA analyses was $P<0.0036$, reflecting Bonferroni correction for 14 factor comparisons. † *P* values adjusted for age, race, sex, body mass index, number of diseased coronary arteries, history of diabetes, hypertension, dyslipidemia, smoking, glomerular filtration rate, insulin resistance, and batch. ‡ Pairwise comparisons for factors significant at Bonferroni corrected threshold test for significant between-group differences. *P* values for factors significant at nominal threshold of $P<0.05$ are reported for exploratory purposes. *P* values reflect between-group pairwise contrasts generated from the fully adjusted ANCOVA procedure. § Values are least square means, adjusted for all 12 covariates. Standard error of the mean is provided beneath each value. Abbreviations: HFpEF indicates heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HF, heart failure; ANCOVA, analysis of covariance; BCAA, branched-chain amino acids; C, carbon chain length.

TABLE S7: Adjusted Metabolite Factor Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls, Controlling for Insulin Resistance but NOT Diabetes*

Factor	Description	ANCOVA	Pairwise Comparisons‡			Metabolite Factor Mean Values§		
		P-value †	HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=263)	HFpEF (N=273)	No-HF (N=183)
1	Medium-chain acylcarnitines	0.18						
2	Long-chain dicarboxyl-acylcarnitines	0.0008	0.002	0.008	1.00	0.305 (0.136)	0.026 (0.137)	0.020 (0.145)
3	Short-chain dicarboxyl-acylcarnitines	0.12						
4	Long-chain acylcarnitines	<0.0001	0.0001	<0.0001	0.004	0.470 (0.155)	0.083 (0.156)	-0.271 (0.165)
5	Ketones and related metabolites	0.36						
6	C8-C10 acylcarnitines	0.03	1.00	0.02	0.20	0.186 (0.132)	0.112 (0.132)	-0.060 (0.140)
7	BCAA and related metabolites	0.008	0.04	0.02	1.00	0.055 (0.151)	-0.171 (0.151)	-0.235 (0.161)
8	Various amino acids	0.03	0.06	0.11	1.00	-0.155 (0.129)	0.028 (0.130)	0.033 (0.138)
9	Short-chain acylcarnitines	0.91						
10	Asparagine, aspartate, 3-hydroxyisovaleryl / malonyl carnitine	0.17						
11	Histidine, arginine, tigylcarnitine, 3-hydroxylinoleyl / hexadecadienedioyl carnitine	0.01	1.00	0.009	0.09	-0.377 (0.111)	-0.318 (0.112)	-0.148 (0.119)
12	Valine, glutamine, glutamate	0.02	0.13	0.02	1.00	-0.422 (0.148)	-0.241 (0.148)	-0.147 (0.158)
13	Alanine, proline, free fatty acids	0.02	0.40	0.02	0.52	0.130 (0.137)	0.004 (0.138)	-0.128 (0.146)
14	Docosanoyl-carnitine	0.07						

* Statistical significance in omnibus ANCOVA analyses was $P<0.0036$, reflecting Bonferroni correction for 14 factor comparisons. † *P* values adjusted for age, race, sex, body mass index, number of diseased coronary arteries, hypertension, dyslipidemia, smoking, glomerular filtration rate, insulin resistance, and batch. ‡ Pairwise comparisons for factors significant at Bonferroni corrected threshold test for significant between-group differences. *P* values for factors significant at nominal threshold of $P<0.05$ are reported for exploratory purposes. *P* values reflect between-group pairwise contrasts generated from the fully adjusted ANCOVA procedure. § Values are least square means, adjusted for all 11 covariates. Standard error of the mean is provided beneath each value. Abbreviations: HFpEF indicates heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HF, heart failure; ANCOVA, analysis of covariance; BCAA, branched-chain amino acids; C, carbon chain length.

TABLE S8: Adjusted Individual Metabolite Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls, Controlling for History of Diabetes and Insulin Resistance

Metabolites		ANCOVA	Pairwise Comparisons†			Mean Concentration in µM‡		
Structure	Trivial Name	*	HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=263)	HFpEF (N=273)	No-HF (N=183)
C16	Palmitoyl-carnitine	<0.0001	0.003	<0.0001	0.0004	0.105 (0.036)	0.097 (0.030)	0.084 (0.026)
C18:2	Linoleyl-carnitine	<0.0001	<0.0001	<0.0001	0.04	0.100 (0.047)	0.083 (0.040)	0.073 (0.028)
C18:1	Oleyl-carnitine	<0.0001	<0.0001	<0.0001	0.006	0.185 (0.077)	0.160 (0.070)	0.137 (0.053)
C18	Stearoyl-carnitine	<0.0001	0.13	<0.0001	0.007	0.049 (0.017)	0.046 (0.015)	0.041 (0.017)
C16:1-OH/ C14:1-DC	3-hydroxy-palmitoleoyl-carnitine or cis-5-tetradecenedioyl-carnitine	<0.0001	0.006	<0.0001	0.06	0.012 (0.006)	0.010 (0.005)	0.009 (0.004)
C20:4	Arachidinoyl-carnitine	<0.0001	0.0003	<0.0001	0.69	0.010 (0.006)	0.008 (0.005)	0.008 (0.004)

* *P* values for multivariate ANCOVA, adjusted for age, race, sex, body mass index, number of diseased coronary arteries, and history of diabetes, hypertension, dyslipidemia, smoking, glomerular filtration rate, batch, and lipoprotein insulin resistance score (LP-IR). † *P* values reflect between-group pairwise contrasts generated from the fully adjusted ANCOVA. ‡ Values are unadjusted mean concentrations. Standard deviation is provided beneath each value. Abbreviations: HFpEF indicates heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HF, heart failure; ANCOVA, analysis of covariance; C, carbon chain length.

TABLE S9: Adjusted Individual Metabolite Means and Comparisons Between HFpEF, HFrEF, and No-HF Controls, Controlling for Insulin Resistance but NOT Diabetes

Metabolites		ANCOVA*	Pairwise Comparisons†			Mean Concentration in µM‡		
Structure	Trivial Name		HFrEF vs HFpEF	HFrEF vs No-HF	HFpEF vs No-HF	HFrEF (N=263)	HFpEF (N=273)	No-HF (N=183)
C16	Palmitoyl-carnitine	<0.0001	0.003	<0.0001	0.0005	0.105 (0.036)	0.097 (0.030)	0.084 (0.026)
C18:2	Linoleyl-carnitine	<0.0001	<0.0001	<0.0001	0.04	0.100 (0.047)	0.083 (0.040)	0.073 (0.028)
C18:1	Oleyl-carnitine	<0.0001	<0.0001	<0.0001	0.009	0.185 (0.077)	0.160 (0.070)	0.137 (0.053)
C18	Stearoyl-carnitine	<0.0001	0.13	<0.0001	0.007	0.049 (0.017)	0.046 (0.015)	0.041 (0.017)
C16:1-OH/ C14:1-DC	3-hydroxy-palmitoleoyl-carnitine or cis-5-tetradecenedioyl-carnitine	<0.0001	0.007	<0.0001	0.046	0.012 (0.006)	0.010 (0.005)	0.009 (0.004)
C20:4	Arachidinoyl-carnitine	<0.0001	0.0003	<0.0001	0.85	0.010 (0.006)	0.008 (0.005)	0.008 (0.004)

* *P* values for multivariate ANCOVA, adjusted for age, race, sex, body mass index, number of diseased coronary arteries, hypertension, dyslipidemia, smoking, glomerular filtration rate, batch, and lipoprotein insulin resistance score (LP-IR). † *P* values reflect between-group pairwise contrasts generated from the fully adjusted ANCOVA. ‡ Values are unadjusted mean concentrations. Standard deviation is provided beneath each value. Abbreviations: HFpEF indicates heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HF, heart failure; ANCOVA, analysis of covariance; C, carbon chain length.

Table S10. Plasma LCAC Means for Primary and Additional Cohorts*						
	HFrEF		HFpEF		No-HF	
Metabolite	Primary Analysis (N=273)	HF-ACTION Trial (N=453)	Primary Analysis (N=263)	RELAX Trial (N=161)	No-HF (N=180)	CATHGEN Overall (N=3653)
C16	0.105 (0.04)	0.081 (0.03)	0.097 (0.03)	0.099 (0.030)	0.084 (0.03)	0.082 (0.026)
C18:2	0.099 (0.05)	0.055 (0.03)	0.084 (0.04)	0.080 (0.035)	0.072 (0.03)	0.070 (0.033)
C18:1	0.185 (0.08)	0.120 (0.05)	0.161 (0.07)	0.138 (0.055)	0.137 (0.05)	0.150 (0.066)
C18	0.050 (0.02)	0.044 (0.01)	0.047 (0.02)	0.043 (0.013)	0.041 (0.02)	0.041 (0.018)
C16:1-OH/ C14:1-DC	0.011 (0.01)	0.007 (0.003)	0.010 (0.005)	0.009 (0.004)	0.009 (0.004)	0.008 (0.004)
C20:4	0.010 (0.01)	0.006 (0.004)	0.008 (0.01)	0.008 (0.004)	0.007 (0.004)	0.007 (0.005)
Values are unadjusted means in uM with standard deviation below.						

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