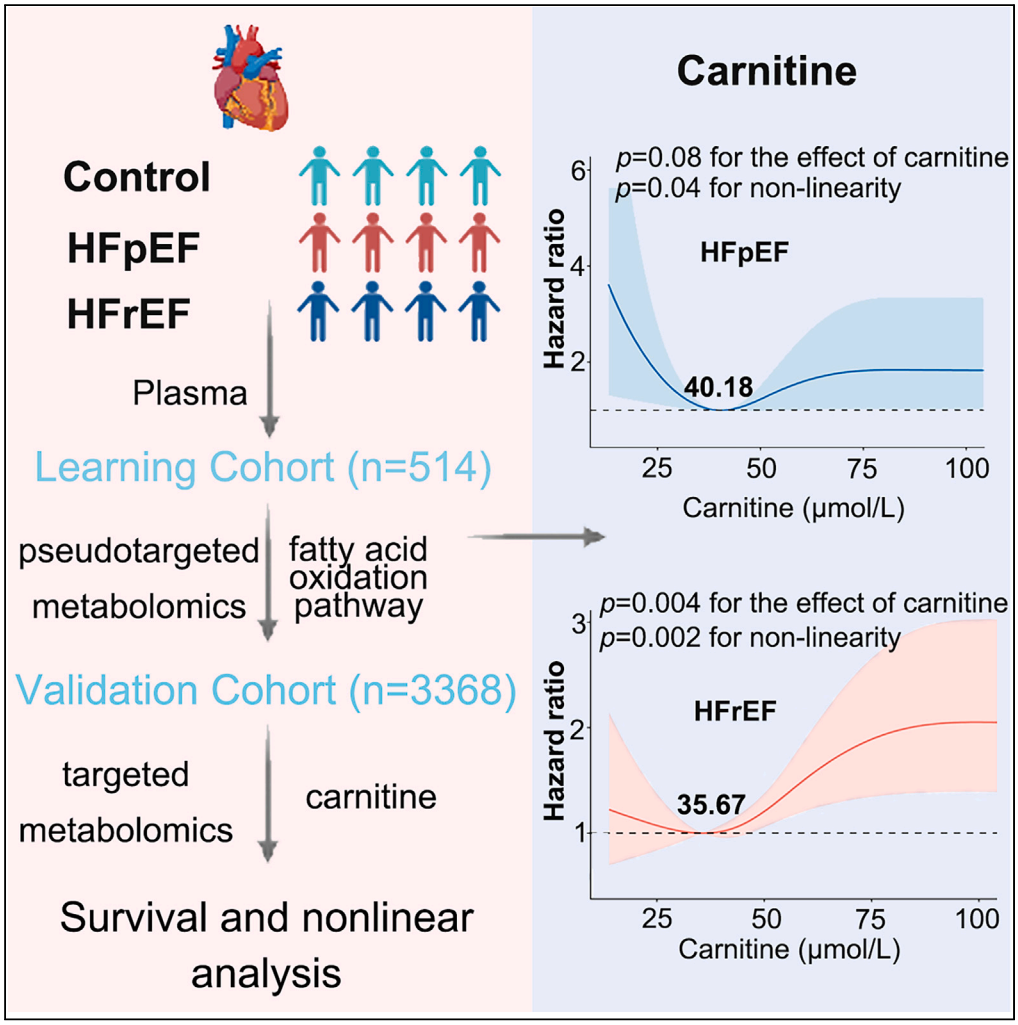
iScience

CellPress
OPEN ACCESS

Article

Carnitine is a friend in HFpEF and foe in HFrEF



Huiqing Wang, Haoran Wei, Mingming Zhao, ..., Yansong Guo, Dao Wen Wang, Lemin Zheng

zhaomingming@pku.edu.cn (M.Z.)
dwwang@tjh.tjmu.edu.cn (D.W.W.)
zhengl@bjmu.edu.cn (L.Z.)

Highlights

HFpEF and HFrEF patients have different carnitine metabolism profiles

Increasing plasma carnitine levels raises cardiovascular risk in HFrEF patients

Both low and high plasma carnitine levels are linked to higher cardiovascular risk in HFpEF

Wang et al., iScience 27, 111018
October 18, 2024
https://doi.org/10.1016/j.isci.2024.111018

iScience

CellPress
OPEN ACCESS

Article

Carnitine is a friend in HFpEF and foe in HFrEF

Huiqing Wang,[1,8] Haoran Wei,[2,8] Mingming Zhao,[3,8,*] Junfang Wu,[2] Min Fei,[4] Nan Lin,[4] Rui Zhan,[5] Qingyuan Liu,[6] Qi Zhang,[1] Xiaodong Yao,[7] Yufei Wu,[1] Wenxin Shan,[1] Hongtu Cui,[3] Liang Ji,[1] Bing Pan,[1] Lu Fang,[1] Yujie Zhu,[1] Xin Li,[1] Yansong Guo,[4] Dao Wen Wang,[2,*] and Lemin Zheng[1,6,9,*]

SUMMARY

Heart failure (HF) is a global concern, particularly HF with preserved ejection fraction (HFpEF), lacking effective treatments. Understanding the differences of metabolic profiles between HFpEF and HFrEF (heart failure with reduced ejection fraction) patients is crucial for therapeutic advancements. In this study, pseudotargeted metabolomics was employed to analyze for disparities of plasma metabolic profiles between HFpEF and HFrEF in two cohorts: discovery (n = 514) and validation (n = 3368). Plasma-free carnitine levels were significant changed in HF patients. A non-linear and U-shaped (for HFpEF) or J-shaped (for HFrEF) association between circulating free carnitine levels and the composite risk of cardiac events were observed. Interestingly, HFpEF patients with low free carnitine ($\leq$40.18 μmol/L) displayed a poorer survival, contrasting with HFrEF where higher levels ($\geq$35.67 μmol/L) were linked to poorer outcomes, indicating distinct metabolism pathways. In conclusion, these findings offer insights into HFpEF metabolic profiles, suggesting potential therapeutic targets.

INTRODUCTION

Heart failure (HF) is categorized based on the left ventricular ejection fraction (LVEF) into HF with reduced LVEF (<40%; HFrEF) and HF with preserved LVEF (≥50%; HFpEF). HF is a complex clinical syndrome.[1] HFpEF affects nearly half of all HF patients, impacting over 13 million adults worldwide. Patients with HFpEF exhibit symptoms similar to those with HFrEF, and they also experience high rates of hospitalization, morbidity, and mortality.[2] Neurohormone-targeted drug therapy has shown beneficial effects on HFrEF outcomes.[3] However, the complexity of HFpEF, including its clinical presentation and associated comorbidities, complicates the development of effective treatments.[4] A significant obstacle in treatment development is the inadequate understanding of the pathogenesis and pathophysiology of HFpEF. Therefore, studying the metabolic profiles of HFrEF and HFpEF is beneficial for understanding the differences between the two, and further elucidating the molecular mechanisms of HFpEF and identifying new therapeutic targets are crucial gaps in cardiovascular medicine.[5]

The heart has exceptionally high energy demands, with approximately 95% of its energy derived from mitochondrial oxidative metabolism and the remaining 5% from glycolysis. The primary substrates for mitochondrial oxidative metabolism are fatty acids and glucose, with about 70% of the energy generated through free fatty acid metabolism.[6] Currently, HF is considered a systemic multi-organ syndrome fundamentally driven by metabolic failure. The failing heart is often described as a "fuel-depleted engine".[7,8] These metabolic alterations include a reduction in fatty acid oxidation rates, partially compensated by an increase in glucose utilization, contributing to the progression of myocardial dysfunction. Recently, there has been growing interest in the overall metabolic landscape, including oxidative stress, inflammation, and mitochondrial dysfunction. For instance, a study have shown that, compared to HFrEF(n = 30), HFpEF (n = 38) myocardial tissues exhibit lower levels of fatty acid metabolites, tricarboxylic acid cycle intermediates, and branched-chain amino acid metabolites, differences that are not observed in plasma.[9] Zhao et al. found that supplementing with carnitine, a crucial metabolite in fatty acid metabolism, can reverse N,N,N-trimethyl-5-aminovaleric acid (TMAVA) - induced myocardial hypertrophy.[10] However, perhaps due to limitations in sample size and other factors, there may be inconsistencies in the metabolic profile research results of HF patients.[9–12] Therefore, conducting metabolic profile

[1]The Institute of Cardiovascular Sciences and Institute of Systems Biomedicine, School of Basic Medical Sciences, State Key Laboratory of Vascular Homeostasis and Remodeling, NHC Key Laboratory of Cardiovascular Molecular Biology and Regulatory Peptides, Beijing Key Laboratory of Cardiovascular Receptors Research, Health Science Center, Peking University, Beijing 100191, China
[2]Division of Cardiology, Department of Internal Medicine and Hubei Key Laboratory of Genetics and Molecular Mechanisms of Cardiological Disorders, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[3]Department of Cardiology and Institute of Vascular Medicine, Peking University Third Hospital, Beijing, China
[4]Department of Cardiology, Shengli Clinical Medical College of Fujian Medical University, Fujian Provincial Hospital, Fuzhou 350001, P.R. China
[5]Research Center for Cardiopulmonary Rehabilitation, University of Health and Rehabilitation Sciences Qingdao Hospital (Qingdao Municipal Hospital), School of Health and Life Sciences, University of Health and Rehabilitation Sciences, Qingdao, China
[6]Beijing Tiantan Hospital, China National Clinical Research Center for Neurological Diseases, Advanced Innovation Center for Human Brain Protection, The Capital Medical University, Beijing 100050, China
[7]School of Pharmacy North Sichuan Medical College, Nanchong, China
[8]These authors contributed equally
[9]Lead contact
*Correspondence: zhaomingming@pku.edu.cn (M.Z.), dwwang@tjh.tjmu.edu.cn (D.W.W.), zhengl@bjmu.edu.cn (L.Z.)
https://doi.org/10.1016/j.isci.2024.111018

studies in large queues can help to more accurately understand the metabolic status of two types of HF and provide a basis for further mechanism research.

Metabolomics analysis techniques can simultaneously quantify various molecular intermediates from multiple major bioenergetic pathways, making them highly suitable for studying metabolic profiles in HF patients.[13–15] As an alternative to untargeted methods, a novel strategy called pseudo-targeted metabolomics offers high sensitivity, specificity, and excellent quantification. This approach can monitor hundreds to thousands of metabolites using dynamic multiple reaction monitoring (MRM) and ensures high data quality,[16] and the pseudo-targeted metabolic profiling, using UHPLC-HRMS (ultrahigh-performance liquid chromatography - high-resolution mass spectrometry), provides abundant metabolic information and ensures comprehensive coverage of the metabolome. So, the pseudo-targeted method is suitable for large-scale sample analyses. To address the utility of this approach in assessing HF, we evaluated the metabolic spectrum differences between HFpEF and HFrEF using an improved pseudo-targeted method (Figure 1A). Our primary aim is to identify metabolic abnormalities in HFpEF and to determine the differential pathways altered between HFpEF and HFrEF.

RESULTS

Baseline characteristics of the participants

We used two cohorts in discovery and validation, including control, HFpEF, and HFrEF groups, respectively. The discovery population (n = 514) for modified pseudo-targeted metabolomics included 203 control samples, 155 patients with HFpEF, and 156 patients with HFrEF (Table 1). The median (25th to 75th percentile) age was 61 years. Compared with control subjects, individuals with HF had a greater prevalence of hypertension, diabetes, CHD, and hyperlipidemia (all $p < 0.001$). Additionally, patients with HF exhibited higher levels of BUN and creatinine than control subjects, but lower HDL (all $p < 0.001$). However, compared with the control group, TC and TG levels showed an increasing trend in HFpEF, while they showed a decreasing trend in HFrEF (all $p \leq 0.01$).

An expanded cohort was used as a validation cohort (n = 3368), consisting of 1000 controls, 955 HFrEF patients, and 1413 HFpEF patients (Table 2). The median (25th to 75th percentile) age was 59 years. Compared with control subjects, individuals with HF had a greater prevalence of smoking, hypertension, diabetes, CHD, and hyperlipidemia (all $p < 0.001$). Additionally, patients with HF exhibited higher levels of BUN and creatinine than control subjects, but lower HDL (all $p < 0.001$). Although there were differences in TC, TG, and LDL levels among the control, HFpEF, and HFrEF groups, it was observed that the changing trends in HFpEF and HFrEF compared with the control group were different. Consistent with the discovery cohort, these results suggest that lipid metabolism pathways differ between HFpEF and HFrEF.

Metabolic spectrum analysis of heart failure based on pseudo-targeted metabolomics technology

In the learning population, a quality control sample containing an equal mix of the samples to be analyzed was prepared to gather comprehensive metabolite information via UHPLC-HRMS. A total of 2359 MRM transitions were selected from the untargeted metabolic profiles in both the positive and negative ion modes. The extracted ion chromatograms of these MRM transitions are shown in Figure S1, with 1178 transitions accurately detected in the samples. A multivariate data analysis was conducted to compare the HF and control group metabolomes. An unsupervised principal component analysis revealed a separation trend among the three groups (Figure 1B). Furthermore, a supervised orthogonal partial least squares discriminant analysis improved the clustering segregation between HFpEF and HFrEF, achieving distinct separation (Figures 1C and 1D).

The differentiation between HFpEF and HFrEF prompted the identification of potential metabolite biomarkers that contributed to metabolomic diversity. To determine the disrupted metabolic pathways in HFpEF, a metabolomic pathway analysis was based on the Kyoto Encyclopedia of Genes and Genomes Pathway Database. Figure 1E lists all disrupted metabolic pathways in HFpEF and HFrEF, with the top potential pathways being arachidonic acid metabolism and fatty acid metabolism. A recent study found significantly lower levels of epoxyeicosatrienoic acid in patients with HFpEF than in controls, indicating a dysfunctional arachidonic acid metabolic pathway.[17] Consequently, we focused on changes in fatty acid metabolism.[10] In the enriched beta oxidation of very long chain fatty acids metabolic pathway, the metabolite L-carnitine was involved and played a significant role.

Methodological validation of LC-MS-based quantitative detection of carnitine

L-carnitine plays a crucial role in energy metabolism and has garnered increasing attention for its potential in the prevention and treatment of cardiovascular diseases, although it remains controversial.[18] Therefore, this study aimed to further quantify carnitine levels in the plasma of a large cohort, focusing on HF patients, especially those with HFpEF and HFrEF. We developed an LC-MS-based quantitative detection method, and the MRM chromatograms of carnitine and its internal standard in plasma are shown in Figure S2. To ensure the stability and reliability of the quantitative detection method, we conducted rigorous methodological validation.[19] Validation of the analytical method was performed by assessing linearity, sensitivity, precision, stability, and recovery. A detailed validation summary, including acceptance criteria, as shown below.

Linearity and sensitivity

Establish calibration curves using calibration standards of different concentrations ranging from 0 to 200 μmol/L. The corresponding parameters of the regression curve are shown in Table S1. The obtained linearity is sufficient, with a correlation coefficient value higher than or equal to 0.9996. The limit of quantification (LOQ) is 31.25 pmol/L (Figure S3).

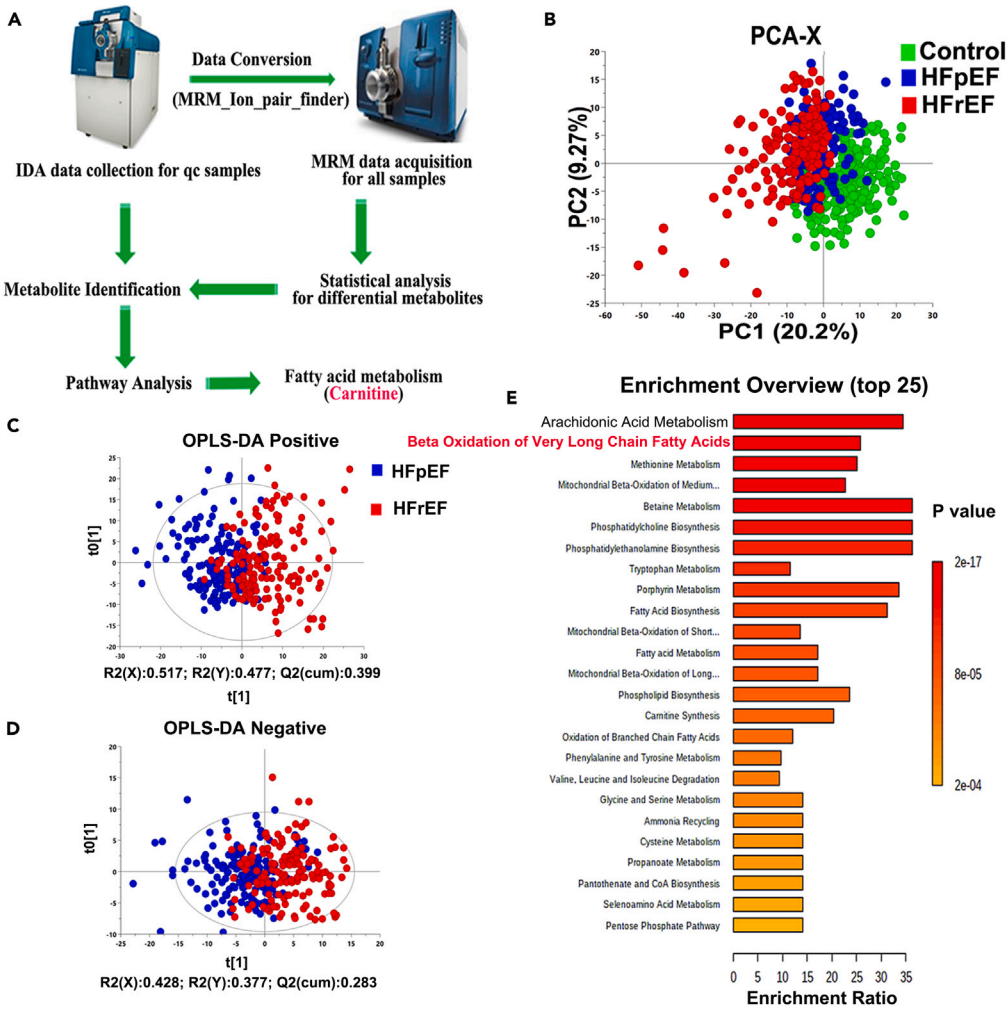


Figure 1. Metabolic differences of carnitine between heart failure and control groups were explored based on an improved pseudotargeted metabolomics technology

(A) The improved pseudotargeted metabolomics technology research process.

(B) Principal component analysis of the control, HFpEF, and HFrEF groups.

(C and D) Supervised orthogonal partial least squares discriminant analyses (OPLS-DA) of HFpEF and HFrEF in positive and negative ion modes separately.

(E) Pathway enrichment analysis between HFpEF and HFrEF.

Recovery

Table S2 summarizes the extraction recovery rates of carnitine at three different concentrations. The extraction recovery rate is 99~101.27%, with an average recovery rate of 100.2%. Therefore, the extraction recovery rate of our method meets the acceptance criteria.

Precision

Instrument precision. As shown in Table S3, the RSD of carnitine concentration for the same sample after six consecutive injection tests is 0.94%.

Method precision. As shown in Table S3, six independent samples were tested and analyzed, and the intra day and inter day precision of carnitine concentration were 2.47% and 3.44%, respectively. These results indicate that our approach is repeatable and reliable in the study.

Stability. We found that carnitine was very stable after three freeze-thaw cycles, with a relative standard deviation of 5.37% in its quantitative results. The detailed results are shown in Table S1, indicating that carnitine has sufficient freeze-thaw stability.

The results above demonstrated that our quantitative method is stable and accurate, suitable for the quantification of carnitine in large clinical cohorts.

Table 1. Demographic and clinical characteristics of control and HF groups in learning population

	All (*n* = 514)	Control (*n* = 203)	HFpEF (*n* = 155)	HFrEF (*n* = 156)	P-value
Demographic characteristics					
Age (years)	61 [52; 68]	61 [52; 68]	61 [52; 68]	62 [51; 69]	0.903
Sex: Female	28.0%	30.5%	29.0%	23.7%	0.341
Smoking	38.5%	36.0%	39.4%	41.0%	0.600
Systolic pressure (mmHg)	129 [117; 145]	130 [120; 143]	134 [120; 150]	124 [111; 140]	0.002
Diastolic pressure (mmHg)	79 [70; 88]	77 [69; 86]	80 [70; 88]	80 [70; 89]	0.162
Heart Rate (bpm)	76 [68; 86]	75 [65; 80]	72 [65; 82]	86 [75; 100]	<0.001
Medical history					
History of hypertension	67.1%	33.5%	86.5%	91.7%	<0.001
History of diabetes	22.8%	5.91%	32.3%	35.3%	<0.001
History of CHD	32.3%	2.96%	58.7%	44.2%	<0.001
History of hyperlipidemia	18.3%	5.91%	34.8%	17.9%	<0.001
Laboratory measurements and echocardiography					
TC (mmol/L)	3.88 [3.25; 4.59]	3.92 [3.30; 4.47]	4.05 [3.44; 4.76]	3.66 [3.06; 4.53]	0.010
TG (mmol/L)	1.12 [0.78; 1.69]	1.15 [0.80; 1.68]	1.27 [0.84; 1.91]	1.00 [0.72; 1.38]	0.002
HDL (mmol/L)	0.98 [0.83; 1.17]	1.06 [0.90; 1.25]	0.93 [0.82; 1.11]	0.91 [0.77; 1.14]	<0.001
LDL (mmol/L)	2.29 [1.78; 2.85]	2.24 [1.84; 2.74]	2.37 [1.79; 3.05]	2.25 [1.64; 2.94]	0.347
BUN (mmol/L)	6.07 [4.64; 7.56]	5.52 [4.36; 6.79]	6.08 [4.69; 7.67]	7.18 [5.77; 9.26]	<0.001
Creatinine (μmol/L)	80 [65; 96]	74 [64; 84]	78 [65; 100]	91 [75; 114]	<0.001
LVEF (%)	60 [38; 66]	65 [62; 69]	62 [57; 66]	33 [27; 38]	<0.001
NT-proBNP (pg/mL)	–	–	307 [136; 1195]	2766 [1560; 7835]	–

Note: Variables are expressed as percentages or medians (interquartile range). Abbreviations: CHD, coronary heart disease; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN, urea nitrogen; LVEF, left ventricle ejection fraction.

Analysis of different metabolic levels of L-carnitine in HFpEF and HFrEF

Previous research disclosed a pronounced decrease in both medium-chain and long-chain acylcarnitines, integral components of fatty acid metabolism, within myocardial tissues of HFpEF and HFrEF patients compared to controls. Interestingly, plasma levels showed either no significant discrepancies or instances of elevation.[9] However, acknowledging limitations in the earlier study, including a relatively modest sample size and a lack of investigation into changes in bioactive free carnitine levels, our research revealed that carnitine levels in HFpEF patients were significantly lower than those in the control group ($p < 0.05$), with no significant difference compared to HFrEF patients in the learning population ($n = 514$) (Figure 2A). Compared to the control group, carnitine levels were significantly reduced in the overall HF population (HFpEF + HFrEF) ($p < 0.05$) (Figure 2B).

Subsequent studies in an independent validation population ($n = 3368$), consisting of 1000 controls, 955 HFrEF patients, and 1413 HFpEF patients, aimed to investigate carnitine changes in HF. In the second population, carnitine levels in HFpEF patients were significantly lower than those in the control ($p < 0.001$) and HFrEF groups ($p < 0.001$) (Figure 2C). Compared to the control group, carnitine levels in the overall HF population (HFpEF + HFrEF) showed a decreasing trend (Figure 2D).

Thus, by using a larger population, we accurately assessed plasma carnitine levels and found that carnitine may serve as a differential metabolic target for HFpEF and HFrEF. This suggests that the two types of HF have distinct lipid metabolism pathways.

Nonlinear analysis

A Cox regression with restricted cubic spline (RCS) analysis revealed a non-linear and U-shaped (Figure 3A) or J-shaped (Figure 3B) association between carnitine levels and the composite risk of cardiovascular death or heart transplantation in HFpEF and HFrEF patients, adjusted for sex, age, smoking status, SBP, CHD, diabetes, HDL, LDL, Cr, and NT-proBNP. RCS is a widely used method for analyzing non-linear relationships between variables and outcomes.[20–22] It essentially acts as a piecewise polynomial but requires continuity and smoothness in the first and second derivatives at each knot, ensuring a good model fit for most scenarios.

These patients were divided into two groups according to the inflection point. HFpEF patients with low carnitine levels (≤40.18 μmol/L), but not those with high carnitine levels (>40.18 μmol/L), were significantly associated with poorer survival. This U-shaped association was robust in the analyses (Figure 3A). In other words, in HFpEF patients, there was no significant increase in cardiac risk associated with plasma carnitine concentrations above 40.18 μmol/L compared to those with low carnitine levels (≤40.18 μmol/L).

Table 2. Demographic and clinical characteristics of control and HF groups in validation cohort

	All (*n* = 3368)	Control (*n* = 1000)	HFpEF (*n* = 1413)	HFrEF (*n* = 955)	P-value
Demographic characteristics					
Age (years)	59 [51; 67]	53 [48; 61]	63 [55; 72]	59 [48; 67]	<0.001
Sex: Female	41.8%	55.6%	40.0%	30.1%	<0.001
Smoking	32.8%	22.6%	34.3%	41.4%	<0.001
Systolic pressure (mmHg)	128 [115; 144]	126 [116; 140]	133 [118; 151]	122 [110; 138]	<0.001
Diastolic pressure (mmHg)	79 [70; 88]	78 [70; 87]	78 [70; 88]	80 [69; 90]	0.395
Heart Rate (bpm)	78 [68; 90]	75 [67; 82]	76 [65; 85]	86 [75; 102]	<0.001
Medical history					
History of hypertension	65.6%	29.8%	83.3%	77.4%	<0.001
History of diabetes	23.1%	6.30%	31.4%	28.7%	<0.001
History of CHD	31.1%	1.70%	52.7%	29.5%	<0.001
History of hyperlipidemia	17.8%	13.0%	21.7%	16.7%	<0.001
Laboratory measurements and echocardiography					
TC (mmol/L)	3.87 [3.25; 4.61]	4.06 [3.49; 4.77]	3.77 [3.18; 4.52]	3.74 [3.13; 4.52]	<0.001
TG (mmol/L)	1.19 [0.85; 1.79]	1.27 [0.87; 1.91]	1.23 [0.86; 1.85]	1.08 [0.79; 1.55]	<0.001
HDL (mmol/L)	0.99 [0.82; 1.20]	1.12 [0.96; 1.33]	0.96 [0.82; 1.14]	0.88 [0.71; 1.10]	<0.001
LDL (mmol/L)	2.30 [1.79; 2.88]	2.39 [1.87; 2.92]	2.19 [1.70; 2.82]	2.37 [1.87; 2.95]	<0.001
BUN (mmol/L)	5.96 [4.67; 7.74]	5.16 [4.24; 6.40]	6.18 [4.82; 7.94]	7.40 [5.70; 10.0]	<0.001
Creatinine (μmol/L)	77 [63; 96]	68 [57; 81]	79 [65; 100]	89 [71; 111]	<0.001
LVEF (%)	60 [38; 66]	65 [61; 69]	63 [57; 67]	31 [25; 36]	<0.001
NT-proBNP (pg/mL)	–	–	535 [137; 2046]	3804 (1745; 8584)	–

Note: Variables are expressed as percentages or medians (interquartile range). Abbreviations: CHD, coronary heart disease; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BUN, urea nitrogen; LVEF, left ventricle ejection fraction.

However, higher carnitine levels were significantly associated with poorer survival in patients with HFrEF. Conversely, those with carnitine levels <35.67 μmol/L were not at an increased composite risk of cardiovascular death or heart transplantation. This J-shaped association was robust in the analyses (Figure 3B). In other words, lower carnitine levels (<35.67 μmol/L) in HFrEF patients might be beneficial, being significantly associated with better survival rates.

Survival analysis

During a median 24-month follow-up, 434 patients (18%) experienced cardiovascular death or underwent heart transplantation (the endpoint). Patients with HF were divided into four groups based on free carnitine quartiles.

Survival curves for HFpEF patients showed that those with plasma free carnitine levels in the lowest (<36.5 μmol/L) and highest (>58.4 μmol/L) quartiles faced a higher risk of cardiac events (Figure 3C). This suggests that carnitine levels within the range of 36.5–58.4 μmol/L are associated with a lower risk of cardiac events in HFpEF patients. Kaplan-Meier analyses indicated an increased risk of cardiovascular death or heart transplantation with higher carnitine levels in HFrEF patients (Figure 3D). This means that lower carnitine levels are linked to a reduced risk of cardiac events in HFrEF patients.

The survival analysis results align with the previous non-linear analysis findings. They recommend specific plasma free carnitine levels for HFpEF and HFrEF patients: (1) HFpEF Patients: A carnitine level range of 36.5–58.4 μmol/L is associated with a lower risk of cardiac events and better survival rates. (2) HFrEF Patients: Lower carnitine levels (<35.67 μmol/L) are linked to a lower risk of cardiac events and better survival rates. This suggests different optimal carnitine levels for improving survival outcomes in HFpEF and HFrEF patients.

DISCUSSION

This study, utilizing a substantial population, revealed a non-linear and U-shaped (Figure 3A) or J-shaped (Figure 3B) association between circulating free carnitine levels and the composite risk of cardiac events. Previous research has commonly reported linear associations between exposure factors and the composite risk of cardiac events.[23,24] A notable strength of this study is that we did not assume a linear relationship between plasma free carnitine levels and the composite risk of cardiac events. Instead, we investigated non-linear associations, adjusting for other influencing factors (such as sex, age, smoking status, SBP, CHD, diabetes, HDL, LDL, Cr, and NT-proBNP) that may affect the

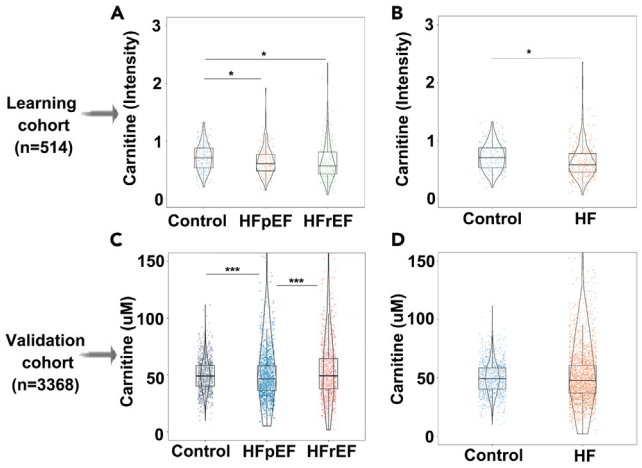


Figure 2. Metabolic differences of carnitine between different groups in learning and validation populations

(A and B) Distribution of carnitine in different groups (learning population). (C, D) Verification of the absolute quantitative analysis of carnitine in different groups (validation population). Data are represented as medians (interquartile range). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

observed associations. Therefore, when conducting clinical trials for HF, it is crucial to consider both the HF subtypes (HFpEF or HFrEF) and the levels of carnitine (low or high).

Carnitine plays a crucial role in the process of fatty acid entry into the mitochondria, and its imbalance can affect fatty acid oxidation.[25] Under normal physiological conditions, the primary source of energy for the heart is oxidative phosphorylation of fatty acids in the mitochondria. However, in HF, there is a shift in the heart's energy metabolism from fatty acid oxidation to glucose.[26,27] Carnitine is emerging as an important factor in cardiovascular diseases, However, previous studies have shown conflicting results regarding plasma levels of L-carnitine in patients with chronic HF. Some studies with small sample sizes ($n < 50$) reported elevated or normal levels of plasma L-carnitine,[28,29] while larger studies ($n = 168$) indicated decreased myocardial carnitine levels with elevated plasma carnitine levels during HF.[23] In our study, we examined a relatively large cohort of HF patients with different etiologies and disease severities. In the discovery population ($n = 514$), plasma carnitine levels were significantly reduced in HF patients compared to controls (Figure 2B). However, in a larger validation population ($n = 3368$), although there was a decreasing trend in plasma carnitine levels in HF patients compared to controls, the difference was not statistically significant (Figure 2D). These findings emphasize the importance of having a larger sample size to ensure more precise estimates.

In our validation population, we observed a downward trend in plasma carnitine levels in HF patients compared to the control group, though the difference was not statistically significant. This suggests that there might be different lipid metabolic pathways in the two subtypes of heart failure (HFpEF and HFrEF), necessitating separate analyses. Several studies have investigated serum free carnitine levels in patients with HFpEF, HFrEF, and non-HF individuals, but the evidence is also contradictory. Some studies report that carnitine levels decrease in HFpEF patients compared to non-HF patients, while others indicate an increase in carnitine levels.[11,30–33] Zhao et al. utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure carnitine levels in serum of HFpEF, HFrEF, HFmrEF, and non-HF patients. They found that HFrEF patients had significantly higher carnitine levels ($p < 0.001$), compared to HFpEF, and all HF patients had higher carnitine levels compared to non-HF individuals.[32] Hage et al. studied 46 HFpEF patients (LVEF $\geq$50%) and 75 HFrEF patients (LVEF <40%) and assessed serum carnitine levels using LC-MS/MS. They observed that HFpEF and HFrEF exhibit distinct metabolic characteristics. However, serum carnitine levels did not differ significantly between HFpEF and HFrEF.[33]

In our study, in the discovery population ($n = 514$), both HFpEF and HFrEF patients showed significant reductions in plasma free carnitine levels compared to controls, whereas there was no significant difference in plasma carnitine levels between HFpEF and HFrEF (Figure 2A). In the validation population ($n = 3368$), HFpEF patients demonstrated significantly lower plasma free carnitine levels compared to controls and HFrEF, while HFrEF patients exhibited significantly higher plasma free carnitine levels compared to HFpEF and no difference compared to controls (Figure 2C). This further confirms our hypothesis that different subtypes of heart failure (HFpEF and HFrEF) involve distinct metabolic pathways that warrant differentiated analysis. Analyzing the conflicting results of existing studies, variations in sample sizes—from relatively small to large—appear to be a contributing factor. This underscores the importance of larger sample sizes for ensuring more accurate estimates, which is a strength of our study.

Currently, studies on carnitine in HF patients not only dispute its levels as mentioned earlier but also debate its effects on the heart. Some research suggests that carnitine has a protective effect on the heart.,[34] while others associate carnitine with coronary heart disease and HF risks.[18] Junko Naito et al. reported that carnitine deficiency can lead to cardiac dysfunction and that carnitine significantly improves HFpEF, as well as left ventricular (LV) systolic function and reduces LV hypertrophy in hemodialysis (HD) patients.[35] Amin Mirrafiei et al., through systematic review and meta-analysis, found that supplementing L-carnitine slightly improves cardiovascular risk factors in adults with type 2 diabetes.[36] However, Yoriko Heianza et al. found a long-term elevation of L-carnitine levels associated with subsequent coronary heart disease incidence, particularly among women with higher red meat intake.[24]

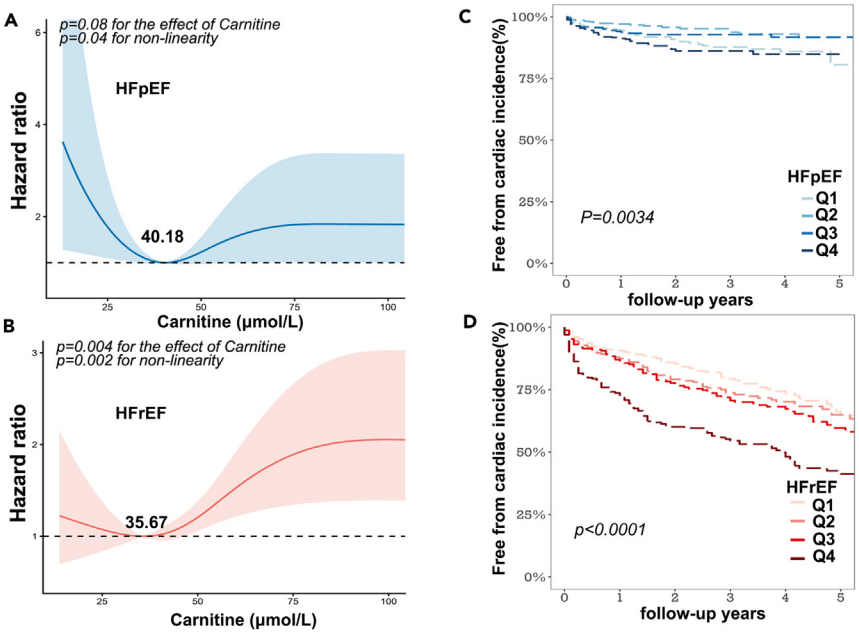


Figure 3. Survival and nonlinear analysis of carnitine between heart failure with preserved ejection fraction (HFpEF) and heart failure with reduced ejection fraction (HFrEF)

(**A** and B) The changing trend of carnitine in the HFpEF and HFrEF groups was characterized by a non-linear statistical analysis.

(C and D) Prognostic analysis of HFpEF and HFrEF.

Currently, the contradictory understanding of carnitine's role compels us to reanalyze its origin and metabolic pathway. Endogenous carnitine biosynthesis involves the rate-limiting step of γ-butyrobetaine (γ-BB) hydroxylation by γ-butyrobetaine hydroxylase (BBOX), leading to carnitine production in both eukaryotes and prokaryotes.[37,38] On the other hand, carnitine can be metabolized into γ-BB exclusively by gut microbiota, which can further break down into trimethylamine (TMA). These represent two distinct metabolic pathways: the former supporting energy metabolism with associated health benefits, and the latter contributing to trimethylamine-N-oxide (TMAO) biosynthesis in the gut, implicated in inflammatory processes underlying diseases like cardiovascular disease (CVD) and metabolic syndrome. Studies suggesting beneficial effects of carnitine have found that treatment with N,N,N-trimethyl-5-aminovaleric acid (TMAVA) significantly reduces plasma and cardiac carnitine levels, indicating the inhibition of fatty acid β-oxidation. Conversely, supplementing with exogenous carnitine alleviates TMAVA-induced cardiac hypertrophy. TMAVA competes with γ-butyrobetaine (γ-BB) for binding to the enzyme BBOX, thereby inhibiting carnitine synthesis. Additionally, TMAVA effectively inhibits the uptake of carnitine by cardiac myocytes through organic cation transporter 2 (OCTN2). These findings highlight the essential role of carnitine in cardiac metabolism.[10] However, another research suggests that carnitine has negative effects. It emphasizes that the metabolism of dietary carnitine by gut microbiota produces trimethyllysine (TML) and trimethylamine (TMA), which in turn enhances the formation of trimethylamine N-oxide (TMAO), especially in individuals with high TMAO levels. Studies have shown that omnivores produce higher levels of TMAO compared to vegetarians or vegans.[39]

In our study, we identified a non-linear relationship between plasma free carnitine levels and the composite risk of cardiac events. Moreover, the role of carnitine in the heart should be discussed based on specific subtypes of heart disease (such as HFpEF and HFrEF, as in this study). For HFpEF patients, lower risk of cardiac events was associated with relatively higher carnitine levels (36.5–58.4 μmol/L) (Figure 3A), correlating significantly with better survival rates (Figure 3C). Conversely, for HFrEF patients, lower carnitine levels (<35.67 μmol/L) were linked to reduced risk of cardiac events (Figure 3B) and better survival rates (Figure 3D). The different effects of carnitine in HFpEF and HFrEF seem to suggest that the two types of HF have different patterns of carnitine production and metabolism. However, the specific mechanisms require further in-depth research in the future. Therefore, it is not definitive whether carnitine is universally beneficial or detrimental in the heart; rather, its effects depend on specific disease subtypes and the non-linear risk thresholds observed in different conditions. This study provides recommended ranges of plasma free carnitine levels for HFpEF and HFrEF patients, offering another type of biological insights particularly for HFpEF, and laying a foundation for precise therapeutic guidance development.

Limitations of the study

This study still has some limitations that need to be addressed.

Study population: The participants in our study were limited to the Han Chinese population in China. It is a single-center prospective cohort study approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology,

Wuhan, China. However, the relatively large study population provides a certain level of accuracy in interpreting the results. Subsequent studies are also ongoing to further validate the findings through multi-ethnic, multi-center cohort studies.

Research findings: This study primarily elucidated the significant role of carnitine metabolism in cardiac energy metabolism, especially in HF diseases, particularly in HFpEF, where effective treatments are currently lacking. Subsequent research will further explore changes in amino acid, lipid, and other metabolite profiles occurring in HF.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, L.Z. (zhengl@bjmu.edu.cn).

Materials availability

Materials generated in this study are available from the lead contact upon request.

Data and code availability

- Data: the raw metabolomics MS data generated in this study have been deposited in the MetaboLights database under accession code MTBLS10993.
- Code: this paper does not report original code.
- All other items: any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

This study was supported by the National High Technology Research and Development Program of China (Grant NO.2020YFA0803700; NO. 2023YFA1800904); the Natural Science Foundation of Beijing, China (Grant NO.L232031; NO.J230039); the National Natural Science Foundation of China (Grants 82104159); the Beijing Municipal Natural Science Foundation (Grant 7242163).

AUTHOR CONTRIBUTIONS

These authors contributed equally: H.Wang, H.Wei, and M.Z. Investigation: M.Z., D.W.W., and L.Z. Designed research studies: M.Z., D.W.W., L.Z.. Conducted experiments and analyzed data: All authors. Drafting of the manuscript: H.Wang, H.Wei, and M.Z. Writing—review and editing: All authors. Funding acquisition: L.Z. and M.Z. Supervision: D.W.W. and L.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Human subjects
  - For HFpEF
  - For HFrEF
  - For non-HF group
- METHOD DETAILS
  - Collection and analysis of pseudotargeted metabolomics data from clinical samples
  - Quantification of plasma free carnitine level by liquid chromatography–mass spectrometry
  - Methodological validation of carnitine quantification using LC-MS
  - Prognostic and nonlinear analysis of plasma free carnitine levels in patients with HFpEF and HFrEF for endpoint events
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.111018.

Received: April 16, 2024
Revised: June 28, 2024
Accepted: September 19, 2024
Published: September 23, 2024

REFERENCES

1. Zhuang, X., Tian, M., Li, L., Xu, S., Cai, M., Yang, X., Qiu, Z., Chai, T., and Chen, L. (2022). Identification of potential hub genes for the diagnosis and therapy of dilated cardiomyopathy with heart failure through bioinformatics analysis. Glob. Transl. Med. *1*, 1–15. https://doi.org/10.36922/gtm.v1i1.104.
2. Hahn, V.S., Knutsdottir, H., Luo, X., Bedi, K., Margulies, K.B., Haldar, S.M., Stolina, M., Yin, J., Khakoo, A.Y., Vaishnav, J., et al. (2021). Myocardial Gene Expression Signatures in Human Heart Failure with Preserved Ejection Fraction. Circulation *143*, 120–134. https://doi.org/10.1161/CIRCULATIONAHA.120.050498.

3. Dunlay, S.M., Roger, V.L., and Redfield, M.M. (2017). Epidemiology of heart failure with preserved ejection fraction. Nat. Rev. Cardiol. *14*, 591–602. https://doi.org/10.1038/nrcardio.2017.65.
4. Schiattarella, G.G., Altamirano, F., Tong, D., French, K.M., Villalobos, E., Kim, S.Y., Luo, X., Jiang, N., May, H.I., Wang, Z.V., et al. (2019). Nitrosative stress drives heart failure with preserved ejection fraction. Nature *568*, 351–356. https://doi.org/10.1038/s41586-019-1100-z.
5. Hunter, W.G., Kelly, J.P., Mcgarrah, R.W., Khouri, M.G., Craig, D., Haynes, C., Ilkayeva, O., Stevens, R.D., Bain, J.R., Muehlbauer, M.J., et al. (2016). 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. J. Am. Heart Assoc. *5*, e003190. https://doi.org/10.1161/JAHA.115.003190.
6. Noordali, H., Loudon, B.L., Frenneaux, M.P., and Madhani, M. (2018). Cardiac metabolism — A promising therapeutic target for heart failure. Pharmacol. Ther. *182*, 95–114. https://doi.org/10.1016/j.pharmthera.2017.08.001.
7. Song, X., Qu, H., Yang, Z., Rong, J., Cai, W., and Zhou, H. (2017). Efficacy and Safety of L-Carnitine Treatment for Chronic Heart Failure: A Meta-Analysis of Randomized Controlled Trials. BioMed Res. Int. *2017*, 6274854. https://doi.org/10.1155/2017/6274854.
8. Yamamoto, T., and Sano, M. (2022). Deranged Myocardial Fatty Acid Metabolism in Heart Failure. Mol Sci *23*, 996. https://doi.org/10.3390/ijms23020996.
9. Hahn, V.S., Petucci, C., Kim, M.S., Bedi, K.C., Wang, H., Mishra, S., Koleini, N., Yoo, E.J., Margulies, K.B., Arany, Z., et al. (2023). Myocardial Metabolomics of Human Heart Failure With Preserved Ejection Fraction. Circulation *147*, 1147–1161. https://doi.org/10.1161/CIRCULATIONAHA.122.061846.
10. Zhao, M., Wei, H., Li, C., Zhan, R., Liu, C., Gao, J., Yi, Y., Cui, X., Shan, W., Ji, L., et al. (2022). Gut microbiota production of trimethyl-5-aminovaleric acid reduces fatty acid oxidation and accelerates cardiac hypertrophy. Nat. Commun. *13*, 1757. https://doi.org/10.1038/s41467-022-29060-7.
11. Ueland, T., Svardal, A., Øie, E., Askevold, E.T., Nymoen, S.H., Bjørndal, B., Dahl, C.P., Gullestad, L., Berge, R.K., and Aukrust, P. (2013). Disturbed carnitine regulation in chronic heart failure - Increased plasma levels of palmitoyl-carnitine are associated with poor prognosis. Int. J. Cardiol. *167*, 1892–1899. https://doi.org/10.1016/j.ijcard.2012.04.150.
12. Ruiz, M., Labarthe, F., Fortier, A., Bouchard, B., Thompson Legault, J., Bolduc, V., Rigal, O., Chen, J., Ducharme, A., Crawford, P.A., et al. (2017). Circulating acylcarnitine profile in human heart failure: a surrogate of fatty acid metabolic dysregulation in mitochondria and beyond. Am. J. Physiol. Heart Circ. Physiol. *313*, 768–781. https://doi.org/10.1152/ajpheart.00820.
13. Li, X., Wu, F., Günther, S., Looso, M., Kuenne, C., Zhang, T., Wiesnet, M., Klatt, S., Zukunft, S., Fleming, I., et al. (2023). Inhibition of fatty acid oxidation enables heart regeneration in adult mice. Nature *622*, 619–626. https://doi.org/10.1038/s41586-023-06585-5.
14. Takeuchi, T., Kubota, T., Nakanishi, Y., Tsugawa, H., Suda, W., Kwon, A.T.J., Yazaki, J., Ikeda, K., Nemoto, S., Mochizuki, Y., et al. (2023). Gut microbial carbohydrate metabolism contributes to insulin resistance. Nature *621*, 389–395. https://doi.org/10.1038/s41586-023-06466-x.
15. Wishart, D.S. (2019). Metabolomics for Investigating Physiological and Pathophysiological Processes. Physiol. Rev. *99*, 1819–1875. https://doi.org/10.1152/physrev.00035.2018.-Metabolomics.
16. Zheng, F., Zhao, X., Zeng, Z., Wang, L., Lv, W., Wang, Q., and Xu, G. (2020). Development of a plasma pseudotargeted metabolomics method based on ultra-high-performance liquid chromatography–mass spectrometry. Nat. Protoc. *15*, 2519–2537. https://doi.org/10.1038/s41596-020-0341-5.
17. Peng, L., Song, Z., Zhao, C., Abuduwufuer, K., Wang, Y., Wen, Z., Ni, L., Li, C., Yu, Y., Zhu, Y., et al. (2023). Increased Soluble Epoxide Hydrolase Activity Positively Correlates with Mortality in Heart Failure Patients with Preserved Ejection Fraction: Evidence from Metabolomics. Phenomics *3*, 34–49. https://doi.org/10.1007/s43657-022-00069-8.
18. Zhao, J.V., Burgess, S., Fan, B., and Schooling, C.M. (2022). l-carnitine, a friend or foe for cardiovascular disease? A Mendelian randomization study. BMC Med. *20*, 272. https://doi.org/10.1186/s12916-022-02477-z.
19. Liu, J., Zhao, M., Zhou, J., Liu, C., Zheng, L., and Yin, Y. (2016). Simultaneous targeted analysis of trimethylamine-N-oxide, choline, betaine, and carnitine by high performance liquid chromatography tandem mass spectrometry. J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. *1035*, 42–48. https://doi.org/10.1016/j.jchromb.2016.09.026.
20. Ho, F.K., Gray, S.R., Welsh, P., Petermann-Rocha, F., Foster, H., Waddell, H., Anderson, J., Lyall, D., Sattar, N., Gill, J.M.R., et al. (2020). Associations of fat and carbohydrate intake with cardiovascular disease and mortality: Prospective cohort study of UK Biobank participants. Br. Med. J. *368*, m688. https://doi.org/10.1136/bmj.m688.
21. Govindarajulu, U.S., Malloy, E.J., Ganguli, B., Spiegelman, D., and Eisen, E.A. (2009). The comparison of alternative smoothing methods for fitting non-linear exposure-response relationships with Cox models in a simulation study. Int. J. Biostat. *5*. Article 2. https://doi.org/10.2202/1557-4679.1104.
22. Wu, L., Shi, Y., Kong, C., Zhang, J., and Chen, S. (2022). Dietary Inflammatory Index and Its Association with the Prevalence of Coronary Heart Disease among 45,306 US Adults. Nutrients *14*, 4553. https://doi.org/10.3390/nu14214553.
23. Yoshihisa, A., Watanabe, S., Yokokawa, T., Misaka, T., Sato, T., Suzuki, S., Oikawa, M., Kobayashi, A., and Takeishi, Y. (2017). Associations between acylcarnitine to free carnitine ratio and adverse prognosis in heart failure patients with reduced or preserved ejection fraction. ESC Heart Fail. *4*, 360–364. https://doi.org/10.1002/ehf2.12176.
24. Heianza, Y., Ma, W., DiDonato, J.A., Sun, Q., Rimm, E.B., Hu, F.B., Rexrode, K.M., Manson, J.E., and Qi, L. (2022). Ten-year changes in plasma L-carnitine levels and risk of coronary heart disease. Eur. J. Nutr. *61*, 1353–1362. https://doi.org/10.1007/s00394-021-02713-x.
25. Pauly, D.F., and Pepine, C.J. (2003). The Role of Carnitine in Myocardial Dysfunction. Am. J. Kidney Dis. *41*, S35–S43. https://10.1016/s0272-6386(03)00115-x.
26. Fillmore, N., Mori, J., Lopaschuk, G.D., and Lopaschuk, G.D. (2014). Mitochondrial fatty acid oxidation alterations in heart failure, ischaemic heart disease and diabetic cardiomyopathy. Br. J. Pharmacol. *171*, 2080–2090. https://doi.org/10.1111/bph.2014.171.issue-8.
27. Lionetti, V., Stanley, W.C., and Recchia, F.A. (2011). Modulating fatty acid oxidation in heart failure. Cardiovasc. Res. *90*, 202–209. https://doi.org/10.1093/cvr/cvr038.
28. Vescovo, G., Ravara, B., Gobbo, V., and Dalla Libera, L. (2005). Inflammation and perturbation of the L-carnitine system in heart failure. Eur. J. Heart Fail. *7*, 997–1002. https://doi.org/10.1016/j.ejheart.2004.11.010.
29. El-Aroussy, W., Rizk, A., Mayhoub, G., Aleem, S.A., El-Tobgy, S., and Mokhtar, M.S. (2000). Plasma carnitine levels as a marker of impaired left ventricular functions. Mol. Cell. Biochem. *213*, 37–41. https://10.1023/a:1007142919941.
30. Wong, C.N., Gui, X.Y., and Rabkin, S.W. (2024). Myeloperoxidase, carnitine, and derivatives of reactive oxidative metabolites in heart failure with preserved versus reduced ejection fraction: A meta-analysis. Int. J. Cardiol. *399*, 131657. https://doi.org/10.1016/j.ijcard.2023.131657.
31. Zordoky, B.N., Sung, M.M., Ezekowitz, J., Mandal, R., Han, B., Bjorndahl, T.C., Bouatra, S., Anderson, T., Oudit, G.Y., Wishart, D.S., et al. (2015). Metabolomic fingerprint of heart failure with preserved ejection fraction. PLoS One *10*, e0124844. https://doi.org/10.1371/journal.pone.0124844.
32. Zhao, H., Shui, B., Zhao, Q., Hu, Z., Shu, Q., Su, M., Zhang, Y., and Ni, Y. (2021). Quantitative Metabolomics Reveals Heart Failure With Midrange Ejection Fraction as a Distinct Phenotype of Heart Failure. Can. J. Cardiol. *37*, 300–309. https://doi.org/10.1016/j.cjca.2020.03.024.
33. Hage, C., Löfgren, L., Michopoulos, F., nilsson, R., Davidsson, P., Kumar, C., Ekström, M., Eriksson, M.J., Lyngå, P., Persson, B., et al. (2020). Metabolomic Profile in HFpEF vs HFrEF Patients. J. Card. Fail. *26*, 1050–1059. https://doi.org/10.1016/j.cardfail.2020.07.010.
34. Wang, Z.Y., Liu, Y.Y., Liu, G.H., Lu, H.B., and Mao, C.Y. (2018). L-Carnitine and heart disease. Life Sci. *194*, 88–97. https://doi.org/10.1016/j.lfs.2017.12.015.
35. Naito, J., Ohashi, H., Ohno, M., Sugiyama, M., Hayakawa, K., Kunishima, A., Takada, N., Kariya, T., Goto, K., Takatsu, H., et al. (2019). Long-Term Levocarnitine Ameliorates Left Ventricular Diastolic as Well as Systolic Dysfunction in Hemodialysis Patients — Multi-Center Study. Circ. Rep. *1*, 508–516. https://doi.org/10.1253/circrep.cr-19-0075.
36. Mirrafiei, A., Jayedi, A., and Shab-Bidar, S. (2024). The Effects of L-Carnitine Supplementation on Weight Loss, Glycemic Control, and Cardiovascular Risk Factors in Patients With Type 2 Diabetes: A Systematic Review and Dose-Response Meta-Analysis of Randomized Controlled Trials. Clin. Therapeut. *46*, 404–410. https://doi.org/10.1016/j.clinthera.2024.03.002.
37. Shekhawat, P.S., Sonne, S., Carter, A.L., Matern, D., and Ganapathy, V. (2013). Enzymes involved in l-carnitine biosynthesis are expressed by small intestinal enterocytes in mice: Implications for gut health. J. Crohns Colitis *7*, e197–e205. https://doi.org/10.1016/j.crohns.2012.08.011.

38. Vaz, F.M., Van Gool, S., Ofman, R., Ijlst, L., and Wanders, R.J. (1998). Carnitine Biosynthesis: Identification of the cDNA Encoding Human γ-Butyrobetaine Hydroxylase. Biochem. Biophys. Res. Commun. *250*, 506–510. https://doi.org/10.1006/bbrc.1998.9343.

39. Koeth, R.A., Wang, Z., Levison, B.S., Buffa, J.A., Org, E., Sheehy, B.T., Britt, E.B., Fu, X., Wu, Y., Li, L., et al. (2013). Intestinal microbiota metabolism of l-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat. Med. *19*, 576–585. https://doi.org/10.1038/nm.3145.

40. Huang, J., Li, C., Song, Y., Fan, X., You, L., Tan, L., Xiao, L., Li, Q., Ruan, G., Hu, S., et al. (2018). ADRB2 polymorphism Arg16Gly modifies the natural outcome of heart failure and dictates therapeutic response to β-blockers in patients with heart failure. Cell Discov. *4*, 57. https://doi.org/10.1038/s41421-018-0058-6.

41. Wei, H., Zhao, M., Huang, M., Li, C., Gao, J., Yu, T., Zhang, Q., Shen, X., Ji, L., Ni, L., et al. (2022). FMO3-TMAO axis modulates the clinical outcome in chronic heart-failure patients with reduced ejection fraction: evidence from an Asian population. Front. Med. *16*, 295–305. https://doi.org/10.1007/s11684-021-0857-2.

42. Hunt, S.A., Abraham, W.T., Chin, M.H., Feldman, A.M., Francis, G.S., Ganiats, T.G., Jessup, M., Konstam, M.A., Mancini, D.M., Michl, K., et al. (2009). 2009 focused update incorporated into the ACC/AHA 2005 guidelines for the diagnosis and management of heart failure in adults: A report of the Aamerican College of Cardiology Foundation/American Heart Association Task Force on practice guidelines: Developed in collaboration with the International Society for Heart and Lung Transplantation. Circulation *53*, e1–e90. https://doi.org/10.1161/CIRCULATIONAHA.109.192065.

43. Yancy, C.W., Jessup, M., Bozkurt, B., Butler, J., Casey, D.E., Drazner, M.H., Fonarow, G.C., Geraci, S.A., Horwich, T., Januzzi, J.L., et al. (2013). 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. *62*, e147–e239. https://doi.org/10.1016/j.jacc.2013.05.019.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
L-Carnitine	Sigma	8.40092
L-carnitine-d9	Sigma	870311P
Formic acid solution	Merck millipore	5438040100
HPLC-grade acetonitrile	Thermo Fisher	046904.K7
Deposited data		
metabolomics raw data	This paper	https://www.ebi.ac.uk/metabolights/MTBLS10993
Software and algorithms		
GraphPad Prism 8	GraphPad	http://www.graphpad.com/scientific-software/prism/
SIMCA 17	Umetrics	http://www.umetrics.com/products/simca
R(3.6.0)	The R Project	https://www.r-project.org
MultiQuant™	SCIEX	https://www.medicalexpo.com.cn/prod/sciex/product-79612-862428.html

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human subjects

The participants enrolled in our study were two cohorts for discovery and validation, including control group, HFpEF, and HFrEF groups, respectively. The learning population (n = 514) for modified pseudotargeted metabolomics included 203 control samples, 155 patients with HFpEF, and 156 patients with HFrEF. An expanded population was used as validation cohort (n = 3368), consisting of 1000 controls, 955 HFrEF patients, and 1413 HFpEF patients. Between 2008 and 2016, patients with HF were recruited from the Department of Cardiology, Tongji Hospital Affiliated with Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Professional investigators were trained to sort the demographic data, clinical characteristics, and laboratory indices into a standardized format. Patients included were contacted on a regular basis by follow-up investigators through telephone and face-to-face interviews. The median follow-up time was 24 months. Non-HF individuals were randomly selected from a population undergoing elective diagnostic coronary angiography in the same department.

The inclusion and exclusion criteria for HF patients have been previously reported.[40,41] The summary is as follows.

For HFpEF

Inclusion criteria

According to the standards of the American College of Cardiology and the American Heart Association[42] NYHA Class II-IV symptoms, preserved ejection fraction (EF $\geq$ 50%), and willingness to participate in the study.

Exclusion criteria

Severe valvular heart disease, heart failure as a primary cause of acute myocardial infarction within the past month, or life expectancy <1 year due to cancer history.

For HFrEF

Inclusion criteria: Compliance with current guidelines,[43] and EF <40%.

Exclusion criteria: Patients with acute coronary syndrome within the past month, severe co-morbid conditions such as severe infection, malignant tumors, or systemic autoimmune diseases, and patients unwilling to participate in the study.

For non-HF group

Inclusion criteria

Coronary artery stenosis $\leq$50%, normal cardiac structure, absence of severe malignant arrhythmias, and willingness to participate in the study.

Exclusion criteria

Severe hepatic or renal dysfunction.

These criteria outline specific conditions necessary for including or excluding patients with HFpEF, HFrEF, and non-HF individuals in clinical studies.

The study was approved by the Ethics Committee of Tongji Medical College and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient enrolled. The ClinicalTrials.gov Identifier is NCT03461107. Full details of the cohorts used are available in Tables 1 and 2.

METHOD DETAILS

Collection and analysis of pseudotargeted metabolomics data from clinical samples

This study employed pseudo-targeted metabolomics detection technology,[16] which combines the advantages of untargeted metabolomics and targeted metabolomics and can serve as an alternative to untargeted methods. The method mainly includes the following steps: (1) Collection of untargeted metabolic profiling data using UHPLC-HRMS of the quality control (QC) samples; (2) Selection of multiple reaction monitoring (MRM) transitions from the untargeted metabolic profiling data; (3) Conversion of MRM transitions from high-resolution mass spectrometry (HRMS) to triple quadrupole mass spectrometry (TQMS), and (4) Validation of the pseudotargeted metabolomics method.

Quantification of plasma free carnitine level by liquid chromatography–mass spectrometry

Blood samples were collected in the fasting state and immediately stored at −80°C until quantitative analysis. For each sample, 20 μL of plasma was mixed with 80 μL of a 5 μmol/L internal standard solution containing d9-carnitine in methanol, in a 1.5 mL tube. Protein in the samples was removed by vortexing for 1 min, followed by centrifugation at 20,000 g at 4°C for 10 min. The supernatant was collected in a mass spectrometry vial with a pre-loaded insert. Calibration curves were prepared using various concentration standards (0–100 μmol/L; 20 μL) processed with the same procedure, with acceptable standard curves having a coefficient of determination (R2) of 0.999.

The supernatant was analyzed by injecting onto a silica column (2.0 mm × 150 mm, Luna 5u Silica 100A; Cat.No. 00F-4274-B0, Phenomenex, Torrance, CA) at a flow rate of 0.5 mL/min using an LC-20AD Shimadzu pump system, and the SIL-20AXR autosampler interfaced with an 6500+ (SCIEX, Foster City, CA, USA). A discontinuous gradient was generated by mixing solvent A (0.1% propanoic acid in water) with solvent B (0.1% acetic acid in methanol) at different ratios, from 2% B linearly to 95% B over 5.0 min, held for 1.0 min, and then back to 2% B.

Electrospray ionization in positive-ion mode with multiple reaction monitoring of precursor and characteristic product-ion transitions of carnitine at m/z 162.1 → 103 and d9-carnitine at m/z 171.1 → 102.8 was used to monitor the analytes. Declustering potentials (DP) and collision energy (CE) were set at 80V and 20.2eV for carnitine, and 98V and 24.9eV for d9-carnitine, respectively. Collision exit potential (CXP) was set at 7V for all analytes. The mass spectrometer was operated under the following conditions: curtain gas (nitrogen), 35; ion spray voltage, 5500V; source temperature, 500°C; ion source gas 1 (zero-grade air), 50; ion source gas 2 (zero-grade air), 55; and collision gas (nitrogen), LOW. Data acquisition and processing were performed using Analyst software 1.7.3 (Sciex).

Methodological validation of carnitine quantification using LC-MS

Linearity and sensitivity

In this study, quantitative analysis was performed using an internal standard method to ensure reproducibility and reliability, with d9-carnitine used as the internal standard. Fluctuations during sample preparation and LC-MS were normalized using the internal standard. Water was selected as the blank matrix, and analytes and internal standards were added at known concentrations. Standard stock solutions containing carnitine and d9-carnitine (1 mg/mL in water) were accurately weighed and prepared. The carnitine stock solution was serially diluted with water to obtain working solutions ranging from 0 to 200 μmol/L (0, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 50, 60, 80, 100, 200 μmol/L). An internal standard solution (5 μmol/L) of d9-carnitine was prepared by diluting its stock solution in methanol. Calibration solutions of 20 μL series concentrations and 80 μL d9-carnitine internal standard solution (5 μmol/L) were mixed and processed as described previously. The supernatant was injected for LC-MS/MS analysis. Quantification was performed using the internal standard method. Calibration samples at each concentration were tested in triplicate. The ratio of carnitine peak area to internal standard peak area was plotted against its known amount on the y axis to generate a calibration curve via linear regression, facilitating determination of carnitine concentrations in the test plasma samples.

The sensitivity of the method was assessed based on the limit of quantification (LOQ) for carnitine, which is determined at a signal-to-noise ratio (S/N) of 10.

Recovery

Working solutions of the above series standard concentrations were added to blank water to achieve concentrations of 20, 50, 80 μmol/L, defined as low, medium, and high concentration levels (n = 3 replicates), followed by sample processing as described previously. Parallel analyses of corresponding standard solution concentrations were conducted. Carnitine recovery was calculated as ([concentration of spiked sample after preparation - concentration of blank sample]/[concentration of spiked sample - concentration of blank sample]) * 100%.

Precision

Instrument precision. Characterization of the relative standard deviation (RSD) of quantification results from six consecutive injections of the same sample (using a sample of intermediate concentration: 50 μmol/L).

Method precision. Evaluation of intra-day precision by preparing six independent samples (n = 6; using a sample of intermediate concentration: 50 μmol/L), and inter-day precision by monitoring eighteen replicates (n = 18) across three different days using the aforementioned samples.

Stability. To assess metabolite stability, samples (using a sample of intermediate concentration: 50 μmol/L) were subjected to three freeze-thaw cycles (−20°C–20°C). Stability was characterized by the RSD of carnitine quantification results across these three freeze-thaw cycles of the sample.

Prognostic and nonlinear analysis of plasma free carnitine levels in patients with HFpEF and HFrEF for endpoint events

We used two cohorts for discovery and validation, including control group, HFpEF, and HFrEF groups, respectively. The learning cohort (n = 514) for modified pseudotargeted metabolomics included 203 control samples, 155 patients with HFpEF, and 156 patients with HFrEF. An expanded population was used as validation cohort (n = 3368), consisting of 1000 controls, 955 HFrEF patients, and 1413 HFpEF patients. The endpoint was the composite of cardiovascular death or heart transplantation. Kaplan-Meier curves with the log rank test were generated to estimate the cumulative percentage of endpoint in free carnitine levels in patients with HFpEF and HFrEF. The Cox regression with restricted cubic spline analysis was used to evaluate the dose-response relationship of carnitine with the risk of HF, and adjusted for sex, age, smoking status, SBP, CHD, diabetes, HDL, LDL, Cr and NT-proBNP.

QUANTIFICATION AND STATISTICAL ANALYSIS

Continuous variables are presented as means (±SD) or medians (interquartile ranges), and differences between groups were analyzed using either one-way analysis of variance or its non-parametric equivalent (Kruskal-Wallis test). Categorical variables are expressed as numbers (%) and compared using the $\chi 2$ test. Kaplan-Meier curves with the log rank test were constructed to estimate the cumulative incidence of endpoints based on free carnitine levels in patients with HFpEF and HFrEF. Cox regression with restricted cubic spline analysis was utilized to assess the dose-response relationship between carnitine levels and the risk of heart failure, adjusting for sex, age, smoking status, systolic blood pressure, coronary heart disease, diabetes, HDL and LDL cholesterol levels, creatinine, and NT-proBNP. Statistical analysis was performed using SPSS version 25.0 (IBM Corp, Armonk, NY) and R (version 3.6.0, Vienna, Austria), with a significance threshold set at 0.05 for two-tailed tests. Statistical significance was determined at the $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ levels.