

Targeted Dried Blood Spot Acylcarnitine and Amino Acid Profiling Reveals a Candidate Metabolic Signature in Pediatric Heart Failure: A Cross-Sectional Case–Control Study

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Abstract

Pediatric heart failure (HF) causes substantial morbidity and mortality, yet the metabolic derangements underlying myocardial energy failure in children remain incompletely characterized. We performed targeted dried blood spot (DBS) acylcarnitine and amino acid profiling in 30 children with HF of mixed etiology and 30 age- and sex-frequency-matched healthy controls, quantifying 44 metabolites by flow-injection tandem mass spectrometry. Seven metabolites were significantly decreased in HF patients ($p < 0.05$, FDR < 0.05): hexanoylcarnitine/adipoylcarnitine (C6DC), tyrosine, octenoylcarnitine (C8:1), glutamate, decadienoylcarnitine (C10:2), proline, and dodecanoylcarnitine (C12), with decreases of 23.9–48.8%. Hydroxyoctadecanoic acid (C18OH) showed the largest overall decrease (57.7%) but did not reach FDR significance. Multivariate modeling using the full metabolite panel achieved excellent, permutation-validated discrimination between groups (PLS-DA accuracy 98%, $Q^2=0.82$; OPLS-DA $R^2Y = 0.835$, $Q^2=0.708$); six metabolites (C6DC, tyrosine, C8:1, glutamate, C10:2, and proline) emerged as the top contributors to this discrimination and are proposed as an exploratory minimal candidate panel, warranting dedicated validation of its own discriminatory performance in larger, independent cohorts. Pathway enrichment highlighted catecholamine biosynthesis, thyroid hormone synthesis, and aromatic amino acid metabolism, without enrichment of fatty-acid β -oxidation or carnitine-synthesis pathways. These findings indicate that pediatric HF involves a coordinated decrease in medium-chain/dicarboxylic acylcarnitines and selected amino acids alongside enrichment of catecholaminergic and aromatic amino-acid pathways, consistent with a myocardial shift away from fatty-acid oxidation. A small, low-cost DBS-based panel may hold promise as a non-invasive adjunct for pediatric HF assessment, pending external validation.

Introduction

Heart failure (HF) in children is a heterogeneous syndrome arising from congenital heart disease, cardiomyopathies, myocarditis, and other structural or functional myocardial disorders, and it remains a leading cause of prolonged hospitalization and mortality in pediatric cardiac and critical care units, with population-based data indicating substantial associated morbidity and resource utilization [1]. Unlike adult HF, which is dominated by ischemic and hypertensive etiologies, pediatric HF reflects a mixture of congenital and acquired substrates, which complicates both diagnosis and biomarker discovery and has prompted dedicated pediatric-specific diagnostic and management guidelines [2].

The failing heart undergoes a well-described metabolic remodeling in which reliance on fatty-acid β -oxidation — normally the dominant source of myocardial ATP — is reduced in favor of glucose and amino-acid utilization, a phenomenon often termed the "fetal metabolic gene program"[3–5]. Acylcarnitines, the products of mitochondrial fatty-acid transport and β -oxidation, and circulating amino acids, which reflect both protein turnover and anaplerotic support of the tricarboxylic acid cycle, are therefore attractive circulating indices of this metabolic shift [6–8]. Targeted mass-spectrometry panels originally developed for newborn screening allow rapid, low-cost, simultaneous quantification of dozens of these metabolites from a small volume of blood [9], and have been increasingly applied to identify

diagnostic and prognostic biomarkers in adult and pediatric HF, congenital heart disease, and single-ventricle physiology [6, 10–14].

In Iran, a national metabolic registry has documented a diverse spectrum of inherited metabolic disorders in the pediatric population [15]. Despite this growing recognition of metabolic disorders in the Iranian pediatric population, targeted metabolomic data in children with HF from Iran and the broader Middle East remain limited. We therefore undertook a targeted dried blood spot (DBS) acylcarnitine and amino acid profiling study comparing children with HF of mixed etiology to healthy controls, with the aims of (1) characterizing the metabolic signature associated with pediatric HF, (2) identifying the biological pathways most strongly perturbed, and (3) deriving a minimal candidate biomarker panel with diagnostic potential.

Results

Participant characteristics

Thirty children with HF (15 male, 15 female) and 30 age- and sex-frequency-matched healthy controls were included in the primary analysis. Among the HF group, median age was 2.8 years (mean 6.0 ± 5.7 years; range 0.07–17.0 years [25 days to 17.0 years]). Mean left ventricular ejection fraction (LVEF) was $36 \pm 12\%$ (range 15–55%). Etiology was heterogeneous: myocarditis ($n = 10$), dilated cardiomyopathy ($n = 8$), congenital heart disease ($n = 1$), combined patent ductus arteriosus/ventricular septal defect ($n = 1$), end-stage renal disease-associated cardiomyopathy ($n = 1$), and hypertrophic cardiomyopathy ($n = 1$); etiology could not be confidently classified from available records for the remaining 8 patients (26.7%; Table 1). This etiological heterogeneity reflects the real-world case-mix of a pediatric HF referral population and is discussed further below.

Table 1
Baseline characteristics of the study population.

Characteristic	HF group (n = 30)	Control group (n = 30)
Sex, male/female	15 / 15	Frequency-matched to cases
Age, years – median (range)	2.8 (0.07–17.0)	Frequency-matched to cases
Age, years – mean $\pm$ SD	6.0 $\pm$ 5.7	—
LVEF, % – mean $\pm$ SD (range)	36 $\pm$ 12 (15–55)	N/A (no cardiac disease)
Etiology of HF, n (%)		
Myocarditis	10 (33.3)	—
Dilated cardiomyopathy	8 (26.7)	—
Congenital heart disease	1 (3.3)	—
PDA + VSD	1 (3.3)	—
ESRD-associated cardiomyopathy	1 (3.3)	—
Hypertrophic cardiomyopathy	1 (3.3)	—
Not classifiable from records	8 (26.7)	—

Controls were healthy children recruited from those presenting to the hospital for a routine health check-up and satisfied the exclusion criteria described in the Methods. Table 1 summarizes the available baseline characteristics.

Univariate metabolite comparison

Of the 44 targeted metabolites, seven were significantly different between HF patients and controls ($p < 0.05$, FDR < 0.05); all seven were decreased in the HF group (Fig. 1a, Table 2). The largest relative decreases were observed for tyrosine (– 48.8%) and hexanoylcarnitine/adipoylcarnitine (C6DC, – 44.3%). Hydroxyoctadecanoic acid (C18OH) showed the largest overall fold-change decrease in the dataset (– 57.7%, FC = 0.423) but did not reach the FDR < 0.05 threshold. Glycine showed a non-significant relative increase ($p \approx 0.1$).

Table 2

Metabolites significantly different between HF and control groups ($p < 0.05$, FDR < 0.05), ranked by t-statistic.

Metabolite	Direction in HF	% change	t-statistic	FDR
C6DC (adipoylcarnitine)	↓ Decreased	-44.3%	-4.643	0.00081
Tyrosine	↓ Decreased	-48.8%	-4.384	0.00101
C8:1 (octenoylcarnitine)	↓ Decreased	-41.2%	-4.045	0.00216
Glutamate	↓ Decreased	-42.4%	-3.511	0.00924
C10:2 (decadienoylcarnitine)	↓ Decreased	-38.8%	-3.008	0.03153
Proline	↓ Decreased	-29.0%	-2.964	0.03153
C12 (dodecanoylcarnitine)	↓ Decreased	-23.9%	-2.823	0.04019
<i>All t-statistics are negative, indicating lower mean concentration in the HF group relative to controls.</i>				

Correlation structure among metabolites

Pearson correlation analysis of all 44 metabolites (Fig. 1b) identified 214 strong pairwise correlations ($|r| > 0.5$, $p < 0.05$). Two major clusters were apparent: a first cluster comprising amino acids (tyrosine, glutamate, proline, alanine, methionine, ornithine, valine, leucine, phenylalanine; $r > 0.7$ among most pairs, e.g., leucine–valine $r = 0.925$, $p < 0.001$), and a second cluster comprising medium- and long-chain acylcarnitines (e.g., C10:1–C10, $r = 0.881$; C12–C14:1, $r = 0.879$; C14:2–C12:1, $r = 0.861$; all $p < 0.001$). Cross-cluster correlations, such as between C6DC and tyrosine ($r = 0.567$, $p < 0.001$), suggest coordinated dysregulation of lipid and amino-acid metabolism. Negative correlations were limited in magnitude ($r \approx -0.2$), indicating largely convergent rather than reciprocal metabolic dysregulation.

Principal component analysis

Unsupervised PCA of all 44 metabolites showed that the first two principal components explained 51.5% of total variance (PC1, 37.8%; PC2, 13.7%; Fig. 1c, d). Samples from the HF group tended to occupy the positive region of PC1, while controls clustered toward negative PC1 values, with partial overlap between groups. Metabolites with the strongest negative PC1 loadings — predominantly medium- and long-chain (hydroxy-)acylcarnitines (C16OH, C18:1OH, C12, C14, C16:1OH) and several amino acids (alanine, proline, methionine, ornithine) — were relatively higher in controls, indicating that PC1 largely captures the degree of impairment in fatty-acid β -oxidation and amino-acid availability.

Supervised multivariate discrimination (PLS-DA and OPLS-DA)

PLS-DA achieved clearer group separation than PCA (component 1, 23.1% variance; component 2, 22.6%; Fig. 2a), with the 95% confidence ellipses for the two groups showing only slight central overlap. A four-component model provided the optimal balance of fit and predictive power (accuracy = 0.98, $R^2=0.91$, $Q^2=0.82$); a fifth component reduced Q^2 , indicating incipient overfitting. Six metabolites had VIP scores > 1.6 on the first PLS-DA component – C6DC, tyrosine, C8:1, glutamate, C10:2, and proline (Fig. 2b, Table 3) – and are proposed as a minimal candidate biomarker panel; the 98% classification accuracy reported above reflects the full 44-metabolite PLS-DA model from which these VIP scores were derived, and the standalone discriminatory performance of this reduced six-metabolite set has not yet been independently modeled.

Table 3
Minimal candidate biomarker panel identified by PLS-DA (component 1 VIP score > 1.6).

Metabolite	VIP score	Metabolic pathway
C6DC (adipoylcarnitine)	2.32	Fatty-acid β -oxidation (medium-chain, dicarboxylic)
Tyrosine	2.22	Aromatic amino acid / catecholamine precursor
C8:1 (octenoylcarnitine)	2.09	Fatty-acid β -oxidation (medium-chain)
Glutamate	1.86	TCA-cycle anaplerosis
C10:2 (decadienoylcarnitine)	1.63	Fatty-acid β -oxidation (medium-chain)
Proline	1.61	TCA-cycle anaplerosis / collagen metabolism

OPLS-DA achieved complete separation between the two groups along the single predictive component (Fig. 2c): all 30 controls fell on one side and all 30 HF patients on the other, with only minimal overlap of the 95% confidence ellipses near the origin. The predictive component accounted for 5.9% of total metabolite (X) variance and the orthogonal component for 36.9%, while the model explained 83.5% of the variance in class membership ($R^2Y = 0.835$) with a cross-validated predictive ability of $Q^2=0.708$ (overall $R^2X = 0.406$). Permutation testing (20 permutations of class labels) confirmed that this performance was not attributable to chance: the highest permuted R^2Y was 0.369 and the highest permuted Q^2 was 0.246, both far below the values of the true model ($p < 0.05$ for both, 0/20 permutations exceeding the observed statistic), and the Q^2 regression line intercepted the y-axis at a negative value (– 0.32), a recognized indicator of a valid, non-overfitted model (Supplementary Fig. S1).

Fourteen metabolites had OPLS-DA VIP scores > 1, led by C6DC (VIP = 2.34), C8:1 (VIP = 2.21), and tyrosine (VIP = 2.16) (Supplementary Table S1). This ranking closely parallels the PLS-DA VIP panel, reinforcing the robustness of the acylcarnitine/amino-acid signature across independent supervised modeling approaches.

Metabolite set enrichment analysis

MSEA using all 44 targeted metabolites identified 39 metabolite sets in total, of which 29 reached $FDR < 0.05$, 31 reached $FDR < 0.1$, and 19 reached a more stringent $FDR < 0.002$ (Fig. 3a, b). The most strongly enriched pathways were catecholamine biosynthesis and thyroid hormone synthesis (enrichment ratio ≈ 23 – 24 ; $FDR < 0.002$), followed by tyrosine metabolism, phenylalanine and tyrosine metabolism, tryptophan metabolism, the glucose–alanine cycle, and glutathione, alanine, and glutamate metabolism (all $FDR < 0.002$). By contrast, none of the fatty-acid β -oxidation or carnitine-synthesis pathways reached statistical significance (FDR ranging from 0.24 for carnitine synthesis to 0.62 for long-chain β -oxidation and overall fatty-acid metabolism), despite the acylcarnitine changes identified in univariate and multivariate analyses.

Correlation between the candidate metabolite panel and left ventricular ejection fraction

To examine whether the candidate six-metabolite panel tracked disease severity, we calculated Spearman rank correlation coefficients between each panel metabolite (C6DC, C8:1, C10:2, glutamate, tyrosine, proline) and LVEF among the 30 HF patients with paired ejection-fraction and metabolite data. None of the six correlations reached statistical significance (all $p > 0.05$; Benjamini–Hochberg FDR -corrected $p \geq 0.91$ for all comparisons), and effect sizes were weak (Spearman $|p|$ range 0.01–0.21; strongest for glutamate, $\rho = -0.21$, $p = 0.27$) (Supplementary Fig. S2, Supplementary Table S3).

Discussion

In this study, targeted metabolomic profiling of dried blood spots identified coordinated reductions in medium-chain and dicarboxylic acylcarnitines (C6DC, C8:1, C10:2, and C12), accompanied by decreased tyrosine, glutamate, and proline levels in children with heart failure (HF) of diverse etiologies. We also observed a marked reduction in long-chain hydroxy-acylcarnitine C18OH. Multivariate modeling demonstrated strong separation between HF and control groups, supported by permutation testing. Metabolite-set enrichment analysis suggested alterations in pathways related to catecholamine biosynthesis, thyroid hormone metabolism, and aromatic amino acid metabolism, while no significant enrichment was observed for fatty acid oxidation pathways. These findings are consistent with metabolic remodeling described in failing myocardium, characterized by altered substrate utilization and impaired metabolic flexibility, which has been extensively reported in adult HF studies [4, 5, 16]. However, because DBS-based metabolite measurements reflect systemic metabolic status rather than direct myocardial metabolism, these findings should be interpreted as an associated metabolic phenotype rather than direct evidence of altered cardiac metabolic flux.

C6DC (adipoylcarnitine/suberoylcarnitine), C8:1 (octenoylcarnitine), C10:2 (decadienoylcarnitine), and C12 (dodecanoylcarnitine) are intermediates of mitochondrial fatty-acid β -oxidation. Their coordinated decrease in HF patients may indicate altered systemic fatty acid utilization and metabolic adaptation associated with HF progression, rather than a primary defect in a specific β -oxidation enzyme. In

contrast to inherited fatty acid oxidation disorders, where substrate accumulation is typically observed, the pattern identified here likely represents a complex response involving substrate availability, mitochondrial regulation, and peripheral metabolic adaptation. Similar alterations in acylcarnitine profiles have been reported in children with single-ventricle physiology and congenital heart disease [6, 11, 17], as well as in adults with HF where changes in acylcarnitine metabolism have been associated with disease severity and recovery after mechanical circulatory support [18].

The absence of significant enrichment of β -oxidation and carnitine synthesis pathways in MSEA further supports the interpretation that the observed changes may represent broader metabolic adaptation rather than a specific pathway blockade. However, the directionality of acylcarnitine alterations in HF remains inconsistent across studies, with both increased and decreased levels reported in pediatric and adult populations. This variability may result from differences in disease etiology, disease stage, medication exposure, nutritional status, and the biological compartment analyzed [18].

The reduction in circulating tyrosine (48.8%) may reflect altered aromatic amino acid metabolism and increased utilization under chronic neurohormonal activation, potentially related to enhanced catecholamine synthesis in HF. This interpretation is supported by the enrichment of catecholamine biosynthesis pathways and previous studies linking tyrosine metabolism with HF severity [7, 19, 20]. Glutamate and proline participate in anaplerotic replenishment of the tricarboxylic acid cycle; their decrease may indicate changes in amino acid utilization as alternative metabolic substrates during cardiac stress, consistent with observations reported in adult and pediatric HF studies [7, 12]. This finding should be interpreted alongside the broader literature on amino-acid catabolism in HF, in which defective branched-chain amino acid handling has similarly been implicated in the progression of ventricular dysfunction [21], suggesting that amino-acid metabolism more broadly, and not only aromatic amino acids, is disturbed in this population.

C18OH showed the largest relative change among the measured metabolites (57.7% reduction); however, it did not remain significant after FDR correction. Therefore, this finding should be considered exploratory and hypothesis-generating rather than a definitive metabolic alteration. Long-chain hydroxy-acylcarnitines have been associated with lipid peroxidation and oxidative myocardial injury in pediatric cardiomyopathy [22, 23], and its pronounced reduction here may reflect altered long-chain fatty acid metabolism in the systemic metabolic response to HF.

The observed clustering of amino acids and acylcarnitines into distinct correlation modules suggests coordinated regulation of metabolic pathways rather than isolated changes in individual metabolites. The strong correlation between leucine and valine is consistent with shared regulation of branched-chain amino acid metabolism, a pathway implicated in cardiac metabolic remodeling [21]. The consistency of differential metabolites across PCA, PLS-DA, and OPLS-DA analyses, particularly C6DC, C8:1, and tyrosine, supports the robustness of the identified metabolic pattern; however, these findings require validation in larger and independent cohorts. Similar acylcarnitine–amino acid correlation patterns and

multivariate separation have been reported in children with single-ventricle HF and congenital heart disease [13].

Metabolite-set enrichment analysis suggested alterations in catecholamine biosynthesis, thyroid hormone metabolism, glucose–alanine cycling, and glutathione metabolism. These findings may reflect systemic responses to cardiac stress, including neurohormonal activation, altered substrate utilization, and oxidative stress adaptation [24, 25]. Enrichment of thyroid hormone synthesis may also reflect a compensatory attempt to sustain basal energy metabolism, although advanced HF is more often associated with low-T3 (euthyroid sick) syndrome, itself an independent predictor of mortality in cardiac patients [26]. Enrichment of the glucose–alanine cycle and glutathione metabolism pathways further supports a shift toward glucose/amino-acid-based energy metabolism and increased oxidative stress, respectively [12]. Taken together with the absence of β -oxidation pathway enrichment, these findings support a model in which pediatric HF is characterized by systemic reprioritization of energy substrate utilization away from fatty acids, mirroring the well-characterized adult HF metabolic phenotype [4, 16] and extending it, for the first time in this cohort, to an Iranian pediatric population.

The six-metabolite exploratory signature including C6DC, tyrosine, C8:1, glutamate, C10:2, and proline demonstrated strong discrimination between HF patients and controls. Nevertheless, this performance should be interpreted cautiously because the study was exploratory, single-center, and lacked an independent validation cohort. Future studies incorporating larger prospective cohorts, HF etiological subgroups, and integration with established clinical markers such as natriuretic peptides and echocardiographic parameters are required to determine the clinical utility of this metabolic signature [3, 6, 11].

Limitations

Several limitations should be acknowledged. The sample size was relatively modest and may have limited detection of smaller effect sizes while increasing the risk of residual overfitting in multivariate models, despite cross-validation and permutation testing. The cross-sectional design precludes assessment of longitudinal changes or causal inference. The cohort included heterogeneous HF etiologies, which, although reflective of real-world practice, may have attenuated etiology-specific signals. Important clinical variables—including functional class, natriuretic peptide levels, medication exposure, nutritional status, and renal function—were incompletely available. In addition, the wide age range raises the possibility of residual age-related confounding. Metabolites were measured in dried blood spots and therefore reflect systemic rather than myocardial metabolism. Finally, this single-center discovery study lacked an independent external validation cohort; the observed discriminatory performance requires confirmation in larger, prospectively enrolled populations before any clinical utility can be inferred.

Conclusions

This targeted dried blood spot metabolomic analysis provides evidence of coordinated alterations in medium-chain/dicarboxylic acylcarnitines and selected amino acids in children with heart failure. The findings are consistent with systemic metabolic remodeling and highlight the potential of a low-cost, minimally invasive platform already used in newborn screening. Given the exploratory nature of this single-center discovery cohort, the proposed six-metabolite signature requires external validation in larger, prospective, and clinically stratified studies before any diagnostic or prognostic application can be considered.

Methods

Study design and setting

This was a cross-sectional case–control study conducted at Children’s Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran, between 2023 and 2024. Metabolite concentrations were measured at a single time point for each participant; no longitudinal follow-up was performed. The study compared dried blood spot (DBS) acylcarnitine and amino acid profiles between children with a confirmed diagnosis of HF and healthy age- and sex-matched controls.

Participants

A total of 30 children with HF and 30 healthy controls were included in the primary analysis, consistent with the pre-specified sample size (below). HF was diagnosed by a pediatric cardiologist on the basis of clinical findings, echocardiography, and, where necessary, complementary cardiovascular imaging. Eligible participants were within the pediatric age range, spanning early infancy to 17 years of age; written informed consent was obtained from the participant and/or legal guardian. Healthy controls were recruited from children presenting to the hospital for a routine health check-up; they had no history of cardiac disease, diabetes, or known metabolic disorders and were frequency-matched to cases for age and sex.

Exclusion criteria (applied to all participants) were: severe acute or chronic illness (including advanced renal failure with $\text{eGFR} < 30 \text{ mL/min/1.73 m}^2$, hepatic cirrhosis, or active malignancy); use of L-carnitine supplements, sodium valproate, or any medication known to affect carnitine metabolism within the preceding three months.

Dried blood spot acylcarnitine and amino acid analysis by LC-MS/MS

Amino acid and acylcarnitine concentrations were measured from dried blood spots (DBS) using a Shimadzu LCMS-8045 triple-quadrupole LC-MS/MS system (Shimadzu, Japan) and the MassChrom® Newborn Screening Kit (non-derivatized method, Chromsystems, Germany) – the same DBS-based assay platform used for national newborn screening programs. Analysis was performed by flow-injection analysis tandem mass spectrometry (FIA-MS/MS) without chromatographic separation, a technique

originally validated for multianalyte newborn screening that permits rapid, simultaneous quantification of a broad panel of metabolites from a single dried blood spot punch [9].

Sample preparation followed the kit protocol: a punch was taken from each dried blood spot card and eluted in methanol containing isotope-labeled internal standards; after extraction, samples were injected directly (injection volume 10 μ L; autosampler temperature 4°C). Instrument conditions were: positive electrospray ionization (ESI+) with multiple reaction monitoring (MRM); interface voltage 2.0 kV; interface temperature 100°C; desolvation-line temperature 250°C; heat block temperature 400°C; nebulizing, drying, and heating gas flows of 3.0, 10.0, and 10.0 L/min, respectively; and collision-induced dissociation (CID) gas pressure 230 kPa. The kit-supplied mobile-phase reagent was delivered isocratically through a single pump line at a programmed flow of 0.02–0.6 mL/min, for a total run time of 1.70 minutes per sample.

Quality control comprised six-point calibration (0.1–100 μ mol/L), two-level QC samples (low, high) in every batch, and isotope-labeled internal standards per the NSK-B kit (Chromsystems). Analytical coefficient of variation was < 15% for all metabolites. Approximately 10% of samples (n = 12) were re-measured in blinded duplicate; the intraclass correlation coefficient was > 0.95. Limits of detection and quantification ranged from 0.05–0.1 and 0.1–0.5 μ mol/L, respectively, depending on the analyte. Dried blood spot cards were stored desiccated at – 20°C and analyzed within 6 months of collection. Raw data were processed with LabSolutions v5.97 (Shimadzu).

Sample size

Sample size was calculated in G*Power v3.1.9.7 [27], assuming a moderate-to-large effect size (Cohen's $d = 0.70$) for the primary acylcarnitine and amino-acid comparisons. With $\alpha = 0.05$ (two-tailed), power = 80%, and a 1:1 allocation ratio, a minimum of 30 participants per group was required.

Statistical and metabolomic analysis

Raw LC-MS/MS data were pre-processed in MetaboAnalyst 6.0 [28]: metabolites with > 15% error across QC samples were excluded, missing values were imputed by k-nearest neighbors, data were normalized to isotope-labeled internal standards, and autoscaling (mean-centered, unit variance) was applied. Univariate comparisons used the independent-samples t-test (or Mann–Whitney U for non-normally distributed variables) with Benjamini–Hochberg false discovery rate (FDR) correction for multiple testing; metabolites were considered significantly different between groups at $p < 0.05$ combined with $FDR < 0.05$. Multivariate analyses comprised principal component analysis (PCA, unsupervised) and partial least squares-discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA, supervised), with model performance assessed by R^2 , Q^2 , classification accuracy under 5-fold cross-validation, and permutation testing (20 permutations). Variable Importance in Projection (VIP) scores were used to rank discriminating metabolites (VIP > 1 considered relevant; VIP > 1.6 considered a high-confidence minimal biomarker panel). Metabolite Set Enrichment Analysis (MSEA, quantitative enrichment analysis) was performed using all 44 targeted metabolites against the reference metabolite-set library. Spearman rank correlation coefficients were calculated between each of the six candidate panel metabolites and LVEF

among HF patients with paired data, with Benjamini–Hochberg false discovery rate correction applied across the six comparisons (SciPy v1.x, Python 3). Analyses were performed in R v4.4.1 (packages mixOmics, ggplot2, pROC, stats), Python 3 (SciPy, pandas), and MetaboAnalyst 6.0.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Tehran University of Medical Sciences (approval code: IR.TUMS.CHMC.REC.1403.094). Written informed consent was obtained from all participants and/or their legal guardians (for participants under 18 years). All demographic, clinical, and laboratory data were coded, and no personally identifying information (name, national ID, or medical record number) was retained in the analytic dataset.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Conceptualization, S.A. and R.M.; Methodology, S.A. and R.M.; Investigation, M.S., MG and R.S.; Data curation, S.A. and M.S.; Formal analysis, S.A. and MGh; Clinical evaluation and confirmation, MG and R.S.; Scientific consultation, F.S., F.A. and P.R.; Supervision, R.M.; Writing – original draft preparation, S.A.; Writing – review and editing, S.A. and R.M.; Project administration, S.A. and R.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

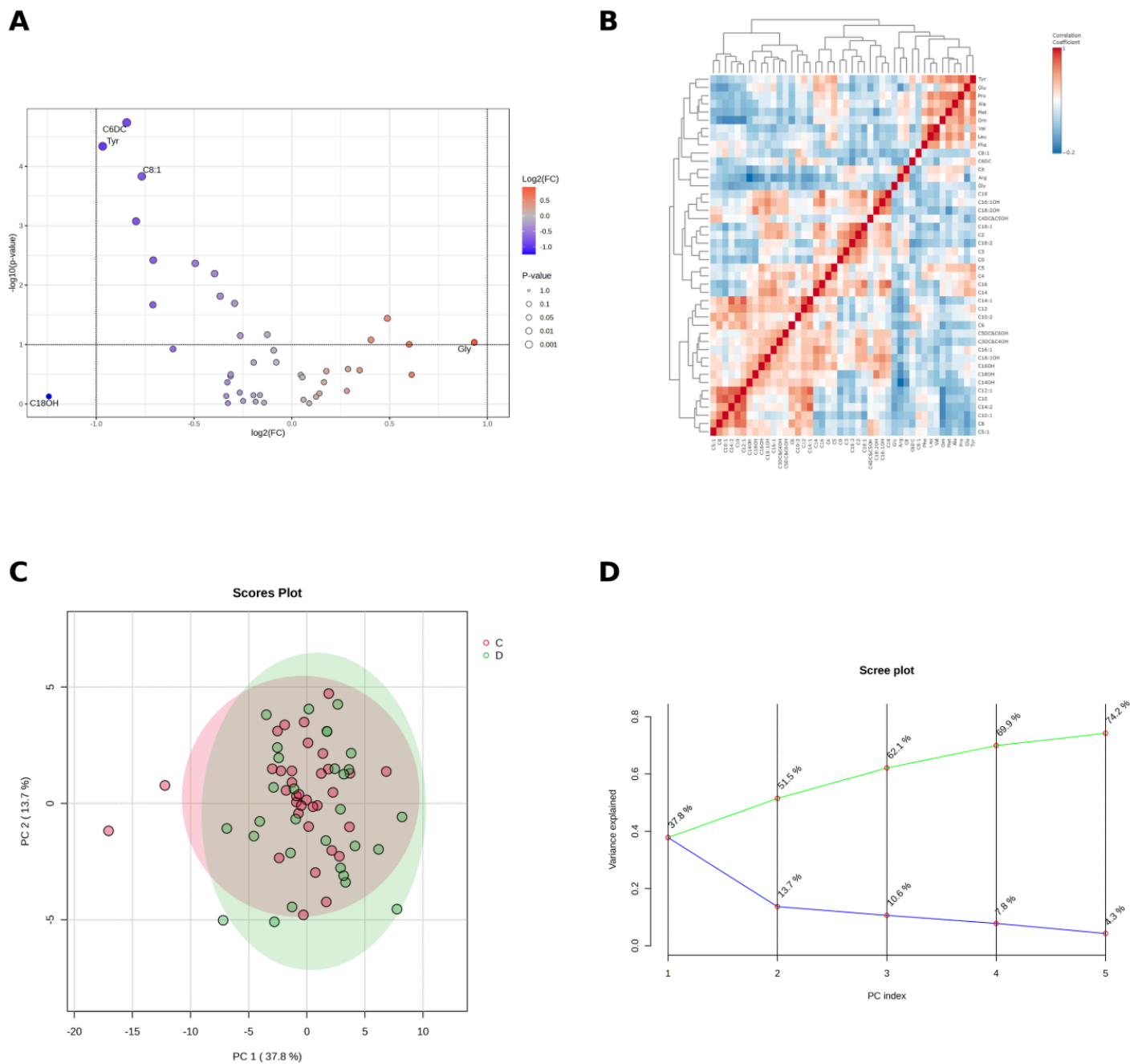


Figure 1

Univariate and unsupervised analysis of the dried-blood-spot metabolome. (a) Volcano plot of all 44 metabolites (x-axis: $\log_2$ fold-change [HF/control]; y-axis: $-\log_{10}$ p-value); the three most significant metabolites (C6DC, tyrosine, C8:1) and the largest-magnitude non-significant metabolites (glycine, C18OH) are labeled; all seven metabolites meeting significance criteria are listed in Table 2. (b) Pearson correlation heatmap with hierarchical clustering of all 44 metabolites, showing separate amino-acid and acylcarnitine clusters. (c) PCA scores plot (PC1 vs PC2; C=control, D=disease/HF; ellipses = 95% confidence regions). (d) PCA scree plot showing variance explained by successive principal components.

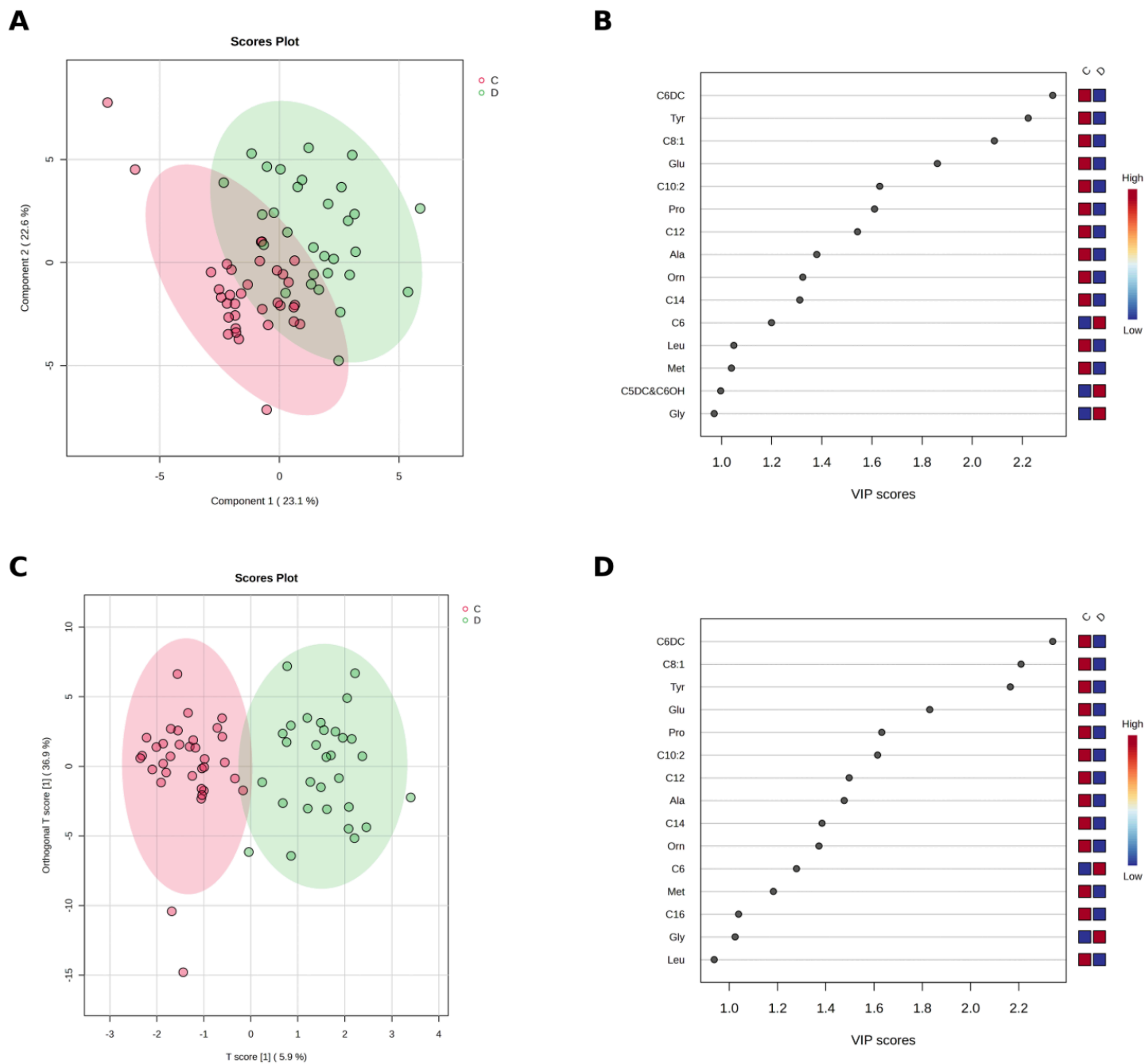


Figure 2

Supervised multivariate discrimination between HF and control groups. (a) PLS-DA scores plot (component 1 vs 2; C=control, D=disease/HF). (b) PLS-DA variable importance in projection (VIP) scores, component 1; colored boxes indicate relative concentration in each group (red=high, blue=low). (c) OPLS-DA scores plot showing complete separation along the predictive component. (d) OPLS-DA VIP scores.

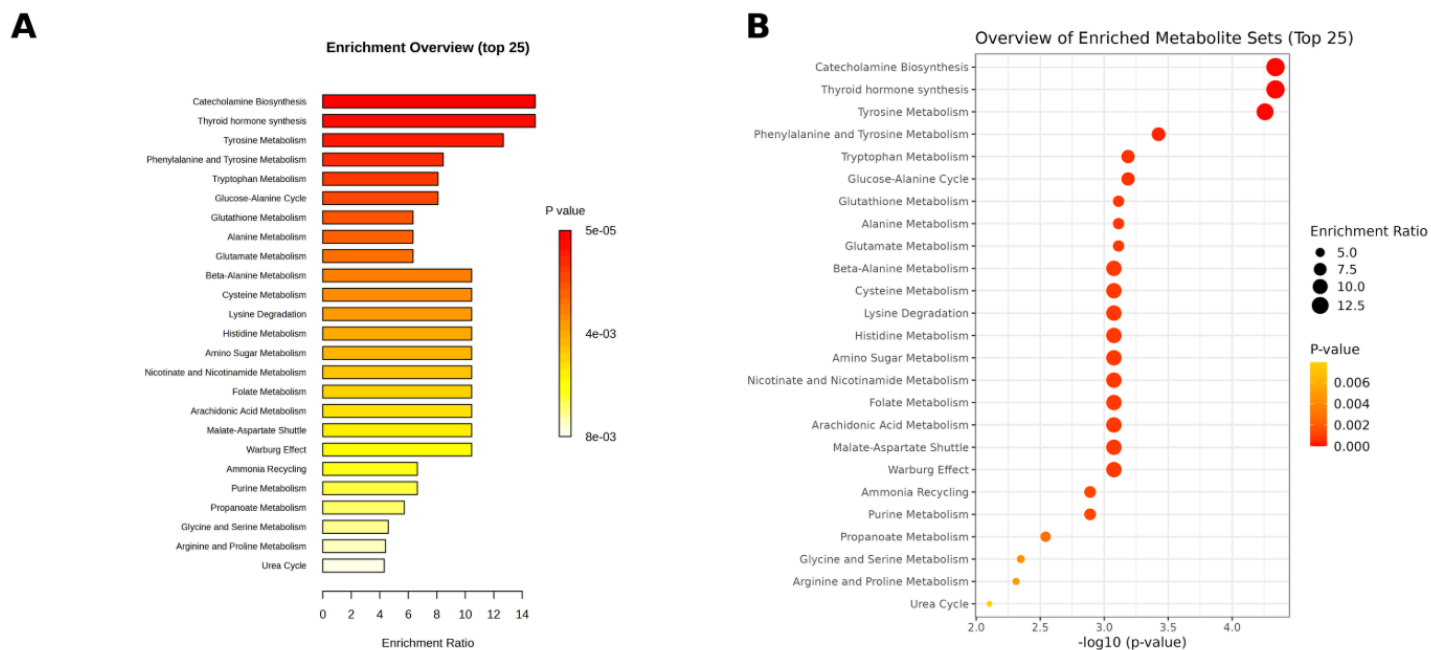


Figure 3

Metabolite set enrichment analysis (MSEA) of all 44 targeted metabolites. (a) Bar chart of the top enriched pathways, ranked by enrichment ratio (color = p-value). (b) Bubble plot of the same pathways (bubble size = enrichment ratio; color = p-value).

Supplementary Files

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