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Untargeted metabolomics reveals distinct biomarkers and metabolic alterations in familial and non-genetic hypercholesterolemia in Saudi patients

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Background: Familial hypercholesterolemia (FH) and non-genetic hypercholesterolemia (HC) are both associated with elevated low-density cholesterol (LDL-C) levels, which increase the risk of cardiovascular disease. However, their underlying metabolic disturbances differ significantly. Untargeted metabolomics offers a powerful approach for identifying disease-specific metabolic signatures and potential biomarkers, thereby contributing to precision medicine applications.

Methods: A high-resolution metabolomics analysis was performed using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) on plasma samples from FH, HC, and healthy Saudi individuals. Differentially expressed metabolites were identified through univariate and multivariate analyses, followed by pathway enrichment analysis using the KEGG database.

Results: Metabolic profiling revealed distinct alterations in bile acid biosynthesis and steroid metabolism pathways in FH. Cholic acid was significantly downregulated, while 17 α -hydroxyprogesterone (17 α -OHP) was significantly elevated in FH. In contrast, HC was characterized by increased uric acid and choline levels, along with dysregulation in oleic acid and linoleic acid metabolism. Notably, both FH and HC groups were dysregulated in Sphinganine, D- α -hydroxyglutaric acid, and pyridoxamine.

Conclusion: This study demonstrates the utility of untargeted metabolomics in distinguishing FH from HC, identifying 17 α -OHP and cholic acid as potential FH biomarkers, while uric acid and choline may serve as HC-specific metabolic markers. These findings provide new insights for personalized interventions,

enhancing disease stratification and therapeutic decision-making between genetic and non-genetic hypercholesterolemia.

KEYWORDS

familial hypercholesterolemia, untargeted metabolomics, KEGG database, biomarkers, cholic acid

1 Introduction

Hypercholesterolemia is a metabolic disorder characterized by elevated low-density lipoprotein cholesterol (LDL-C), contributing significantly to atherosclerosis and cardiovascular diseases (CVDs) (1). Primary hypercholesterolemia includes familial hypercholesterolemia (FH, OMIM #143890), a monogenic disorder caused by variants in genes such as *LDLR*, *APOB*, and *PCSK9* (autosomal dominant), or *LDLRAP1* (autosomal recessive) (2, 3). These genetic alterations impair LDL-C clearance, leading to persistently high cholesterol levels from an early age and an increased risk of premature CVD. The global prevalence of FH is approximately 1 in 250, but it is substantially higher in the Gulf region, around 1 in 112, due to high consanguinity rates (4–7). Despite this, omics-based studies, particularly metabolomics, remain limited in Middle Eastern populations. In parallel, secondary or non-genetic hypercholesterolemia, which is driven by factors such as diet, obesity, and metabolic syndrome, may exhibit clinical features similar to those of inherited forms, making it difficult to distinguish between them using standard lipid profiling (1, 8, 9). Given that genetic variants can directly influence metabolic pathways, individuals with FH are likely to exhibit distinct metabolic signatures compared to those with secondary hypercholesterolemia (10). Understanding these differences is critical for accurate diagnosis, personalized risk assessment, and targeted treatment.

Untargeted metabolomics enables comprehensive profiling of small molecules, offering a powerful approach to identify disease-specific metabolic phenotypes and biomarkers that reflect both genetic and environmental influences on lipid metabolism (11, 12). Among various platforms, untargeted metabolomics using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) offers high sensitivity and broad metabolite coverage, enabling detailed profiling of both the metabolome and lipidome (13, 14). This approach surpasses traditional lipid panels by uncovering disturbances in key metabolic pathways, such as in bile acid biosynthesis, steroid hormone metabolism, and oxidative stress, while simultaneously providing mechanistic insights into cholesterol-related pathologies like atherosclerosis, thereby facilitating biomarker discovery, improving disease classification, and advancing precision medicine strategies (15–17). A recent cross-sectional study conducted, utilizing LC-MS-based untargeted metabolomics, successfully identified unique metabolites associated with different FH forms. The analysis identified seven key discriminatory metabolites, including lithocholic acid (LCA), triacylglycerol TAG 52:2, 3-phenylpropionate, pipercolic acid, 3-indolepropionic acid, isocitric acid, and glycerophosphocholine (GPC) 38:5 (18).

Despite these advances, no studies to date have applied untargeted metabolomics to differentiate genetic and non-genetic forms of

hypercholesterolemia in the Saudi population, which is characterized by unique genetic backgrounds and high rates of early-onset CVD. Therefore, this study aims to apply untargeted high-resolution metabolomics to distinguish between familial and secondary hypercholesterolemia in Saudi patients, identify disease-specific metabolic signatures, and uncover clinically relevant biomarkers. These insights are expected to advance disease classification and support the implementation of precision medicine strategies in hypercholesterolemia management.

Studying hypercholesterolemia in the Saudi population with its unique genetic background and high incidence of early-onset CVD presents a valuable opportunity to fill a key knowledge gap and advance precision medicine strategies tailored to the region. This study aims to apply untargeted high-resolution metabolomics to differentiate familial and non-genetic hypercholesterolemia in Saudi patients, identify specific metabolic signatures, and discover potential biomarkers for clinical use. These insights are expected to support precision medicine by improving disease classification and individualized treatment strategies.

2 Materials and methods

2.1 Study population

This study was approved by the Biomedical Ethics and Research Committee at King Abdulaziz University, Jeddah, Saudi Arabia (Reference Number: 220–22). Families were recruited from the Genetic Dyslipidemia and Familial Hypercholesterolemia Clinic at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The study included three groups of participants: familial hypercholesterolemia (FH), hypercholesterolemia (HC), and healthy controls (Healthy), consisting of both index patients and unaffected family members as controls.

2.2 Sample collection

A 3 mL blood EDTA tube was collected after 10–12 h of fasting, and only individuals without prior lipid-lowering treatment or medication were included. The groups were classified based on LDL-C levels determined through biochemical laboratory testing and genetic testing results (1, 8, 9). Participants were classified based on the results of biochemical and genetic testing, as reported previously (19).

The Healthy group included individuals with LDL-C levels below 100 mg/dL and no detected pathogenic variants in the FH-related genes. The HC consisted of individuals with LDL-C levels ranging from 130 to 159 mg/dL, without confirmed variants in the FH-related genes. The FH group included individuals with LDL-C levels ≥ 190 mg/dL and a confirmed pathogenic variant in the FH-related genes.

2.3 Metabolites extraction

For a plasma separation, blood was centrifuged at $2,000 \times g$ for 15 min at 4°C , and the supernatant was aliquoted (100–200 μL) into a sterile Eppendorf (EP) tube. Plasma samples were then stored at -80°C until further sample processing.

For metabolite extraction, 100 μL of plasma was mixed with 700 μL of extraction solvent containing an internal standard (Methanol: Acetonitrile: Water, 4:2:1, v/v/v). The mixture was vortexed for 1 min and incubated at -20°C for 2 h, then centrifuged at $25,000 \times g$ at 4°C for 15 min. After centrifugation, 600 μL of the supernatant was transferred to a new EP tube and dried using a vacuum concentrator. The dried extracts were reconstituted in 180 μL of Methanol: Water (1:1, v/v), vortexed for 10 min, and centrifuged again at $25,000 \times g$ at 4°C for 15 min to remove any insoluble debris. The final supernatants were transferred to fresh EP tubes for downstream metabolomic analysis.

2.4 Liquid chromatography-mass spectrometry (LC–MS) workflow

The metabolomics analysis was conducted in collaboration with the Beijing Genomics Institute (BGI). Metabolite separation and detection were performed using a Waters UPLC I-Class Plus system (Waters, USA) coupled with a Q Exactive Orbitrap high-resolution tandem mass spectrometer (Thermo Fisher Scientific, USA). Chromatographic separation was carried out using a Waters ACQUITY UPLC BEH C18 column (1.7 μm , 2.1 mm $\times$ 100 mm, Waters, USA), with the column temperature maintained at 45°C .

The mobile phase was prepared based on the ionization mode. In positive ion mode, the mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B), whereas in negative ion mode, the mobile phase contained 10 mM ammonium formate (A) and acetonitrile (B). A gradient elution program was applied with an initial 0 to 1 min 2% B, linearly increasing to 98% B from 1 to 9 min, maintaining 98% B from 9 to 12 min, then reverting to 2% B at 12.1 min, followed by equilibration at 2% B for 12.1 to 15 min. The flow rate was maintained at 0.35 mL/min, and the injection volume was 5 μL .

Mass spectrometric analysis was performed using full scan and tandem MS (MS/MS). The scan range was set from 70 to 1,050 m/z, with a resolution of 70,000 for full MS scans. The Automatic Gain Control (AGC) target was configured to 3×10^6 , with a maximum ion injection time of 100 ms. For MS/MS fragmentation, the top 3 precursor ions were selected per cycle with an injection time of 50 ms, a resolution of 17,500, and the AGC was 1×10^5 . The stepped normalized collision energy was 20, 40, and 60 eV to enhance fragmentation efficiency. Electrospray ionization (ESI) settings were optimized as follows: the sheath gas flow rate was set to 40, and the auxiliary gas flow rate was set to 10. The spray voltage was adjusted to 3.80 kV for positive ion mode and 3.20 kV for negative ion mode. The capillary temperature was maintained at 320°C , while the auxiliary gas heater temperature was set to 350°C .

2.5 Peak area and metabolites identification

The generated mass spectrometry data were imported and processed using Compound Discoverer v3.3 (Thermo Fisher Scientific,

USA)¹. To ensure comprehensive metabolite identification, multiple databases, including BGI Metabolome Database (BMDB), mzCloud, and ChemSpider (HMDB, KEGG, LipidMaps) databases, were used for metabolite peak areas extraction and metabolite identification.

2.6 Data processing

The results file generated from Compound Discoverer v3.3 was imported into MetaX software (20) for data preprocessing and statistical analysis. To minimize technical variability and enhance the reliability of metabolomic data, the following preprocessing steps were implemented. Probabilistic Quotient Normalization (PQN) was applied to normalize metabolite intensities by generating a reference vector based on ion intensity distribution across all samples and adjusting each sample accordingly (21). Additionally, Quality Control-based Robust LOESS Signal Correction (QC-RLSC) was implemented to correct batch effects using local polynomial regression fitting based on QC samples to enhance signal consistency (22). To ensure high-quality and reproducible data for downstream analysis, metabolites with a Coefficient of Variation (CV) > 30% across QC samples were considered unstable and excluded.

The identified substances were classified into different credibility levels based on available matching criteria, including MS1 molecular weight, MS2 fragment spectra, column retention time, and the presence of reference standards. Level 1 represents the highest credibility, where substances are accurately identified using both the standard databases and laboratory data. Level 2 includes substances with a structural formula that matches the standard databases. Level 3 substances partially match the database but require further validation. Level 4 substances are identified solely based on accurate MS1 molecular weight matching the database. Level 5 represents the lowest credibility, with no matches or identification results available in the database. The credibility of identification decreases progressively from Level 1 to Level 5.

2.7 Overall metabolites analysis

To gain more insight into the biological functions and classifications of the identified metabolites from levels 1 to 4. The identified metabolites were analyzed and annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Human Metabolome Database (HMDB). The analysis of identified metabolites was categorized according to their Super Pathway. However, for lipid molecules, the Sub Pathway classification was used instead.

2.8 Differential metabolite screening

Univariate analysis was conducted to assess the statistical significance of differences in metabolite expression between comparison groups, with fold change (FC) calculated as the ratio of metabolite levels and t-tests used for statistical evaluation. Metabolites were considered differentially expressed if they met the threshold of $\text{FC} \geq 1.2$ or ≤ 0.83 and adjusted p -value < 0.05. The $\log_2\text{FC}$ transformation was applied to normalize data

¹ <https://mycompounddiscoverer.com>

distribution and enhance interpretability. Statistical significance was determined using *t*-tests, and adjusted *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg method to control the False Discovery Rate (FDR). The $\log_2$ FC and adjusted *p*-value were used for downstream statistical analysis. The volcano plots were generated to visualize differentially expressed metabolites (DEMs), highlighting significantly upregulated and downregulated metabolites based on their fold changes and adjusted *p*-values. Metabolites with a high absolute $\log_2$ fold change and adjusted *p*-value were considered statistically significant. Heatmaps were generated to illustrate clustering patterns of DEMs between experimental groups.

In multivariate analysis, the orthogonal partial least squares discriminant analysis (OPLS-DA) is used to develop predictive models and identify key metabolites by distinguishing between systematic and orthogonal variance. This enhances classification accuracy and provides a robust evaluation of metabolic distinctions.

2.9 Correlation analysis

Chord diagrams were constructed to evaluate metabolite co-regulation relationships based on the Spearman correlation coefficient ($|r| > 0.8$, $p < 0.05$). These diagrams provide an intuitive visualization of metabolite-metabolite interactions, distinguishing between synergistic (positive correlations) and antagonistic (negative correlations), with color variations representing different correlation patterns. The primary objective of differential metabolite correlation analysis was to assess the consistency of metabolite fluctuations and determine their interdependence.

2.10 Pathway enrichment analysis

Metabolic pathway enrichment analysis of DEMs was performed using the KEGG database to identify significantly altered pathways and interpret biological phenotypes. Pathways with adjusted *p*-value < 0.05 were considered significantly enriched, highlighting key metabolic shifts. To provide a comprehensive view of metabolic alterations, the Differential Abundance (DA) Score method was applied to evaluate cumulative metabolite changes within specific pathways. The DA score ranges from -1 to $+1$, where $+1$ indicates complete upregulation of a pathway, 0 represents no significant change, and -1 denotes complete downregulation. Intermediate values reflect partial upregulation or downregulation based on the proportion of upregulated and downregulated metabolites within the pathway.

3 Results

3.1 Sample description

In this study, 16 fasting plasma samples were analyzed and categorized into three groups: FH ($n = 7$), HC ($n = 4$), and Healthy ($n = 5$). The average LDL-C levels were 367.3 ± 101.8 mg/dL in the FH group. This level was approximately 3.9 times higher than that of the Healthy group (94.6 ± 11.4 mg/dL) and 2.6 times higher than that of the HC group (139.2 ± 4.5 mg/dL). Genetically, all participants in the FH group carried a heterozygous mutation in the *LDLR* gene (c.2416dupG

and c.103C > T), while other groups were negative for FH-related genes. For comparative analysis, three pairwise comparisons were conducted: FH vs. Healthy, HC vs. Healthy, and FH vs. HC to assess metabolic variations across groups. Sample characteristics, including LDL-C measurement, are detailed in the [Supplementary Table 1](#).

3.2 Chromatographic and mass spectrometric analysis

Untargeted metabolomics profiling in both positive and negative ion modes revealed a diverse range of metabolites across the 15-min chromatographic run. As shown in the Base Peak Chromatograms (BPC) ([Supplementary Figure 1](#)), the wide distribution of peaks in both ionization modes confirms broad metabolite coverage and efficient chromatographic separation.

In positive ion mode, prominent features were observed at 7.36, 9.71, and 12.75 min, corresponding to abundant hydrophobic metabolites, while earlier peaks (at 0.88 and 2.67 min) indicated the presence of polar compounds. The negative mode captured a distinct profile of acidic and polar metabolites, with major peaks detected at 9.73, 10.32, and 12.83 min.

3.3 Metabolites detection and identification

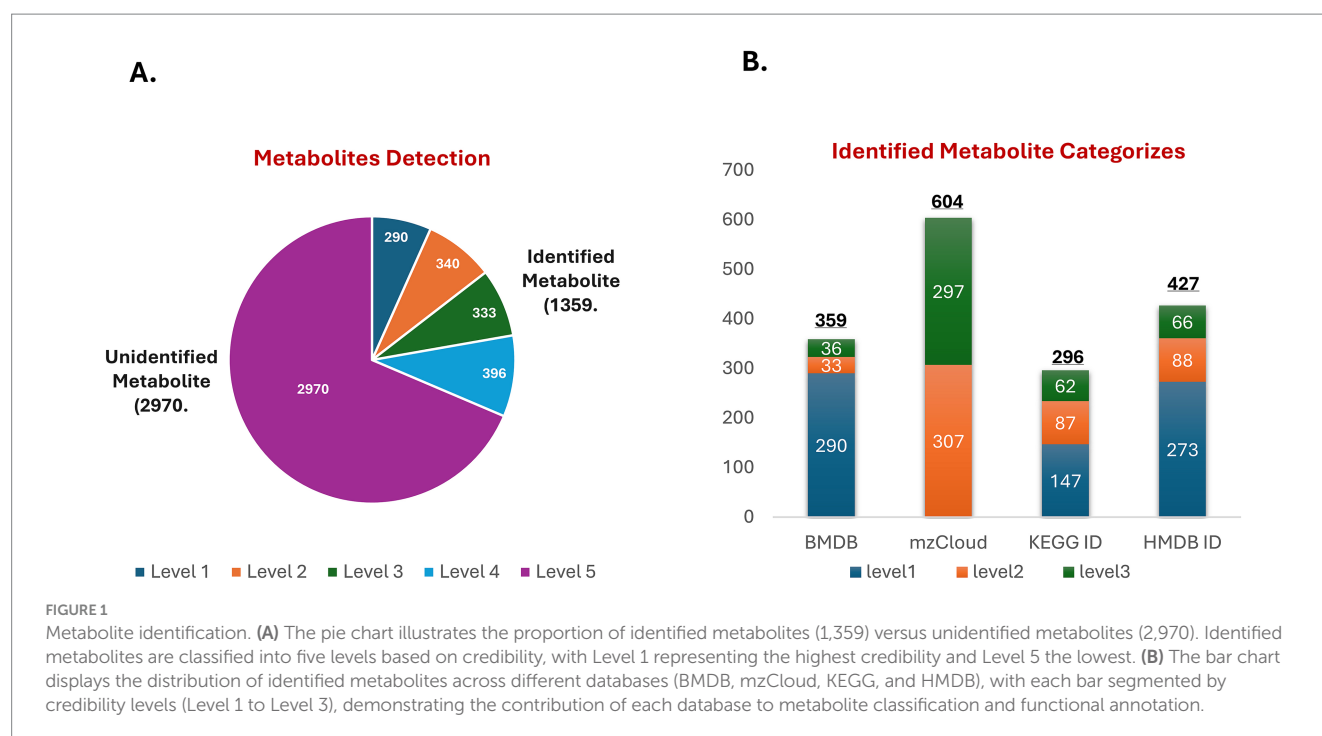
After data preprocessing, a total of 4,850 metabolites were detected, of which 4,329 metabolites had a $CV \leq 30\%$. These metabolites were mapped and classified based on their level of credibility as described in the methods (section 2.6). [Figure 1](#) shows that only 1,359 substances were mapped from Level 1 to Level 4, considered identified, while the remaining metabolites were classified as Level 5 (unidentified). Furthermore, identified metabolites with putative names were annotated using the HMDB and KEGG databases and subsequently classified into four major categories: lipids, phytochemical compounds, biologically active compounds, and others. Among these, lipids ($n = 132$) and amino acids, peptides, and analogues ($n = 64$) represented the two largest classes. Detailed metabolite classifications and KEGG pathway annotations are provided in [Supplementary Figure 2](#).

3.4 Differential metabolites screening and analysis

Univariate analysis across the three groups identified DEMs and revealed distinct distribution patterns ([Figure 2](#)). In the FH vs. Healthy group, 98 DEMs were identified, with 60 upregulated and 38 downregulated. For the HC vs. Healthy group, 175 DEMs were identified, comprising 75 upregulated and 100 downregulated. Finally, in the FH vs. HC group, 83 DEMs were identified, including 38 upregulated and 45 downregulated ([Figure 3](#)). The significant DEMs annotated with HMDB and KEGG IDs were included in the enrichment analysis ([Supplementary Table 2](#)).

We then performed a multivariate statistical analysis using OPLS-DA, which revealed clear ellipses among the FH, HC, and Healthy groups.

The FH vs. Healthy comparison showed a T score of 12.4% and an Orthogonal T score of 13.2%. The HC vs. Healthy comparison had a



T score of 22.8% and an Orthogonal T score of 17.4%. The FH vs. HC comparison showed a T score of 11.7% and an Orthogonal T score of 12.9% (Figure 4).

3.5 DEMs co-regulation relationships

The correlation analysis revealed distinct patterns of metabolite interactions across the three comparison groups (Figure 5). In the FH vs. Healthy comparison (Figure 5A), strong positive intra-class correlations were observed among glycerophospholipids and steroid derivatives, suggesting a tightly regulated lipid metabolic network in individuals with FH. In contrast, negative correlations were noted between glycerophospholipids and amino sugar-related metabolites, indicating a possible dysregulation in membrane lipid signaling and transport. The FH vs. HC analysis demonstrated more divergent correlation patterns, where glycerophospholipids showed both positive and negative correlations with fatty acyls and bile acid derivatives (Figure 5B). These findings may reflect distinct bile acid biosynthesis, lipid remodeling, and oxidative stress response pathways that characterize genetically driven FH compared to HC. In the HC vs. Healthy group, a broader range of negative correlations was found across fatty acyls and glycerophospholipids, consistent with disrupted lipid regulation and systemic metabolic imbalance in HC (Figure 5C). These findings highlight the distinct metabolic network reorganization observed between FH and HC, emphasizing the role of lipid subclass-specific interactions as key contributors to the pathophysiology of different forms of hypercholesterolemia.

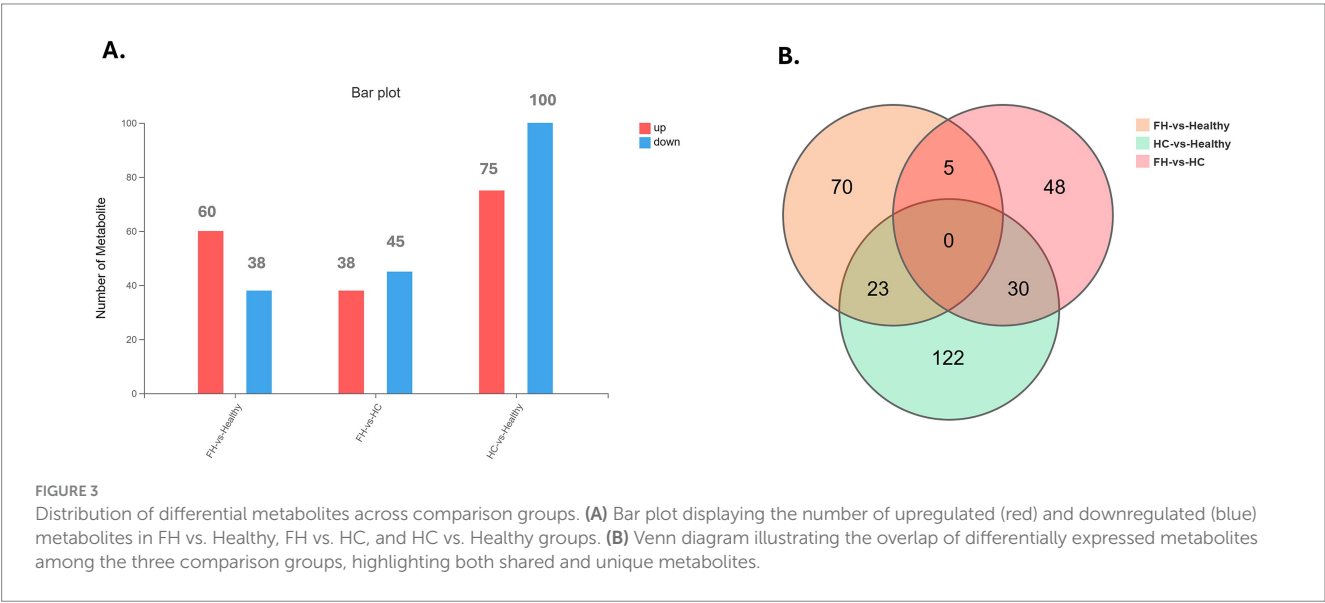
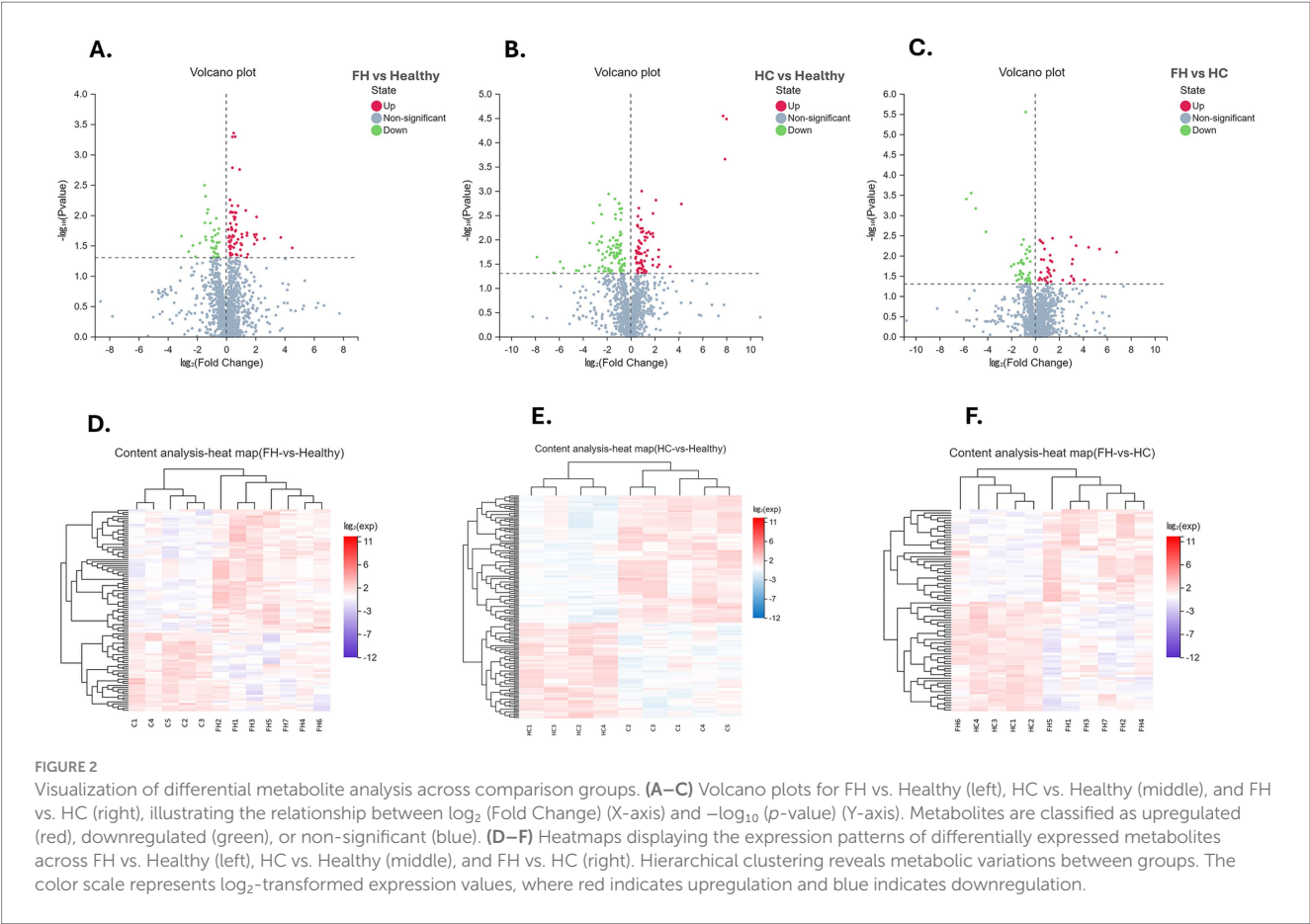
3.6 Pathway enrichment analysis

Metabolic pathway enrichment analysis was performed using the KEGG database to identify significantly altered pathways across the

comparison groups. The top enriched pathways were visualized using a bubble plot in Figure 6, with detailed results presented in Table 1 and Supplementary Table 3.

Shared and unique pathways are illustrated in Figure 6A. The Venn diagram showed the shared metabolites between the comparison groups (Figure 6B). Interestingly, the lysine degradation pathway (KEGG: map00310) was consistently enriched across all three comparative groups. In the FH vs. Healthy comparison, the pathway was significantly altered (adjusted p -value = 0.0499) due to the upregulation of D- α -hydroxyglutaric acid. In the HC vs. Healthy group, this pathway exhibited stronger significance (adjusted p -value = 0.0062) and involved two metabolites: D- α -hydroxyglutaric acid and glutaric acid. However, glutaric acid displayed opposite expression in the two comparisons: it was downregulated in HC vs. Healthy, but upregulated in FH vs. HC, where the lysine degradation pathway remained significant (adjusted p -value = 0.0344). The sphingolipid signaling pathway (KEGG: map04071) and sphingolipid metabolism pathway (KEGG: map00600) were significantly enriched in both FH and HC groups compared to healthy controls. In the FH vs. Healthy comparison, these pathways were enriched with sphinganine (KEGG: C00836) (adjusted p -value = 0.0113 and 0.0124, respectively). In contrast, in the HC vs. Healthy comparison, enrichment was driven by elevated levels of sphinganine and phytosphingosine (adjusted p -value = 0.0260 and 0.0004, respectively).

In the FH vs. Healthy comparison, the cortisol synthesis and secretion pathway (KEGG: map04927) was significantly enriched (adjusted p -value = 0.0136), with upregulation of D- α -hydroxyglutaric acid (Figures 7A,D). In the HC vs. Healthy comparison, significant alterations were observed in the bile secretion pathway (KEGG: map04976, adjusted p -value = 0.0249) and insulin resistance pathway (KEGG: map04931, adjusted p -value = 0.0311), accompanied by upregulation of uric acid, choline, and L-acetylcarnitine. Meanwhile, the fatty acid biosynthesis



pathway (KEGG: map00061, adjusted *p*-value = 0.0311) was downregulated, reflected by decreased oleic and linoleic acid levels (Figures 7B,E).

The FH vs. HC analysis revealed significant enrichment in the primary bile acid biosynthesis pathway (KEGG: map00120, adjusted *p*-value = 0.0359), with downregulation of cholic acid (Figures 7C,F).

4 Discussion

Familial hypercholesterolemia is a metabolic disorder associated with a significantly increased risk of cardiovascular diseases and other health complications (23). Metabolomics, the comprehensive study of metabolites within biological systems, has emerged as a powerful tool in elucidating hyperlipidemic conditions characterized by elevated lipid

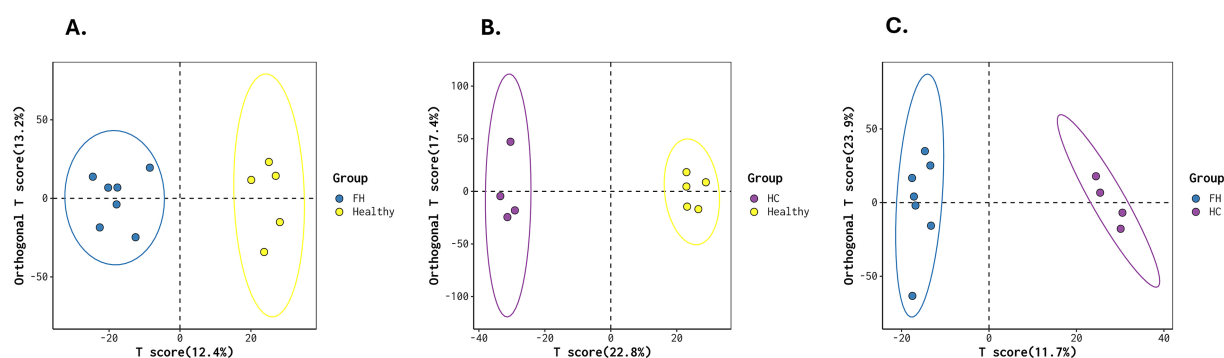


FIGURE 4

OPLS-DA score plots for metabolic differentiation across groups. (A) FH vs. Healthy, (B) HC vs. Healthy, and (C) FH vs. HC, illustrating distinct clustering patterns that highlight metabolic differences between groups. The separation of ellipses indicates variations in metabolic profiles across the groups.

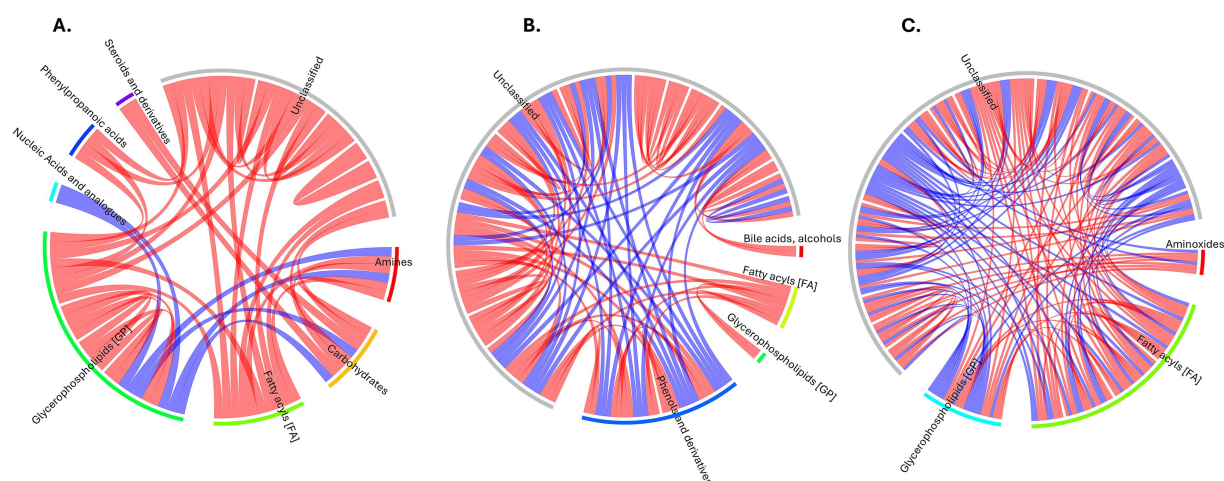


FIGURE 5

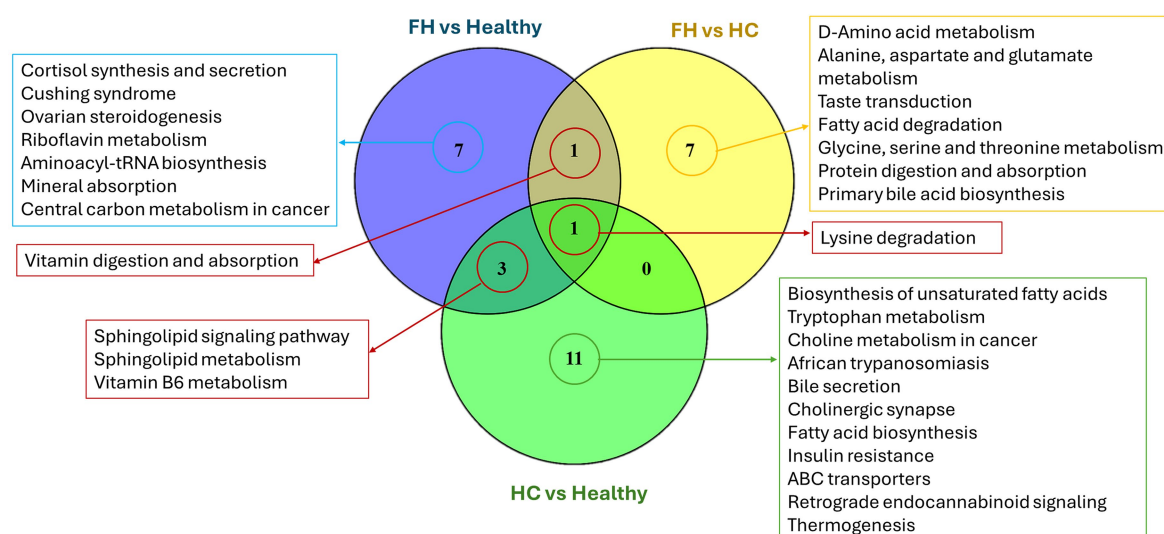
Correlation chord diagrams of metabolite relationships for comparison groups. (A) FH vs. Healthy. (B) FH vs. HC. (C) HC vs. Healthy. Color Key: Red: Positive correlations. Blue: Negative correlations. Connections represent significant correlations ($p < 0.05$) between metabolite pairs.

levels in the bloodstream (24, 25). This approach not only enables the identification of novel biomarkers for early diagnosis but also provides insights into the efficacy of therapeutic interventions (17, 26). In this study, untargeted metabolomics revealed distinct metabolic alterations in both FH patients carrying LDLR variants (c.2416dupG, c.103C > T) and in individuals with HC, compared to healthy controls. KEGG pathway enrichment analysis (adjusted $p < 0.05$) showed significant disruptions in lipid metabolism across both groups. Although both FH and HC are characterized by elevated LDL-C levels, their underlying metabolic mechanisms differ. This distinction is clinically important, as FH is a monogenic disorder caused by inherited variants in lipid-regulating genes, whereas HC typically results from environmental factors, dietary habits, and broader metabolic dysregulation (1, 8, 9).

The FH group, when compared to healthy controls, exhibited significant enrichment in three metabolic pathways, including cortisol synthesis and secretion, Cushing syndrome, and ovarian steroidogenesis, primarily driven by elevated levels of 17α -hydroxyprogesterone (17α -OHP). As cholesterol is the precursor for all steroid hormones, these findings suggest FH caused by LDLR variants may prompt alternative tissues, such as the adrenal glands,

cells can maintain high intracellular cholesterol by increasing cholesterol uptake via HDL pathways via scavenger receptors (27) and under sustained ACTH stimulation (28) which together upregulate steroidogenic enzymes such as CYP17A1 (17α -hydroxylase) (29, 30); consequently, the heightened steroidogenic flux can exceed the capacity of downstream steps like 21 -hydroxylation by CYP21A2, leading to a bottleneck that causes accumulation of precursor steroids, notably 17α -OHP. Previous studies show that Elevated LDL enhances adrenal steroidogenesis by increased ACTH stimulation, commonly associated with metabolic stress in FH, resulting in elevated 17α -OHP levels due to a bottleneck at the CYP21A2 enzyme step (31–33). Clinically, 17α -OHP is a key diagnostic biomarker for congenital adrenal hyperplasia (34–36). In addition, elevated 17α -OHP can lead to an increased risk of cardiovascular (37). These alterations may contribute to broader metabolic and endocrine dysregulation in FH patients, particularly in the context of our study, where the majority of participants were female, suggesting potential sex-specific metabolic responses. The upregulation of 17α -OHP highlights a potential crosstalk between lipid metabolism and adrenal steroidogenesis in FH patients, and this metabolite is considered a secondary metabolite.

A.



B.

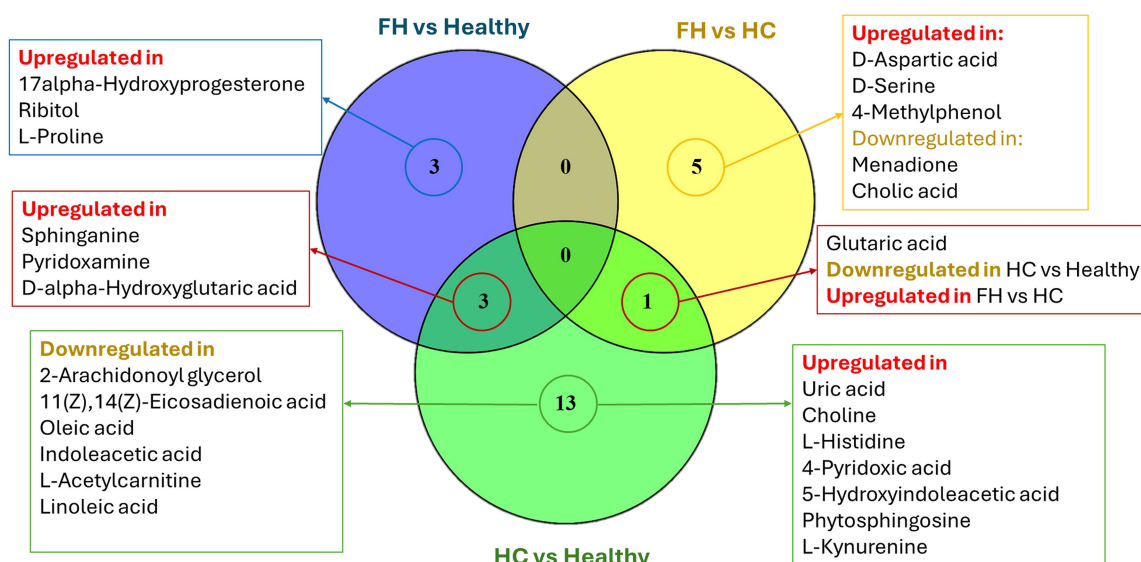


FIGURE 6

Shared and unique pathways and metabolites among comparison groups. (A) Venn diagram showing the number of significant metabolic pathways (adjusted p -value < 0.05) shared and unique among the three pairwise comparisons: FH vs. Healthy, FH vs. HC, and HC vs. Healthy. (B) Venn diagram displaying the significant metabolites contributing to those pathways, categorized by their regulation status (upregulated or downregulated) in each comparison.

On the other hand, comparisons of FH and HC patients revealed a significant dysregulation in primary bile acid biosynthesis, showing that cholic acid levels were significantly downregulated in FH (38, 39). This indicates impaired primary bile acid biosynthesis, likely due to increased intracellular cholesterol that suppresses bile acid synthesis via negative feedback on CYP7A1 (40). Disruption of bile acid metabolism in FH may hinder lipid digestion and feedback regulation, contributing to further cholesterol accumulation (41–43). Moreover, the bile acid impairment observed in familial hypercholesterolemia highlights a significant therapeutic gap. Traditional bile acid sequestrants (BASs), such as cholestyramine, may be insufficient. Emerging biopolymer-based BASs could provide improved FH management by modulating both cholesterol and bile acid metabolic pathways (44–46). These findings indicate that cholic acid has the

potential to serve as a discriminative biomarker between familial and non-genetic forms of hypercholesterolemia.

In the HC group, there was a significant downregulation in the biosynthesis pathways of unsaturated fatty acids (UFAs), including oleic acid, linoleic acid, and 11(Z),14(Z)-eicosadienoic acid, alongside alterations in insulin resistance-related metabolites such as L-acetylcarnitine. It is plausible that these shifts reflect lipid accumulation and impaired fatty acid oxidation, both of which are known to contribute to insulin resistance and metabolic inflexibility (47). These UFAs, particularly monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs), are vital for cholesterol transport and CVD prevention (48–51). Reduced levels may be driven by insulin resistance, which suppresses stearoyl-CoA desaturase-1 (SCD1), the enzyme converting saturated fatty acids into MUFAs, including oleic

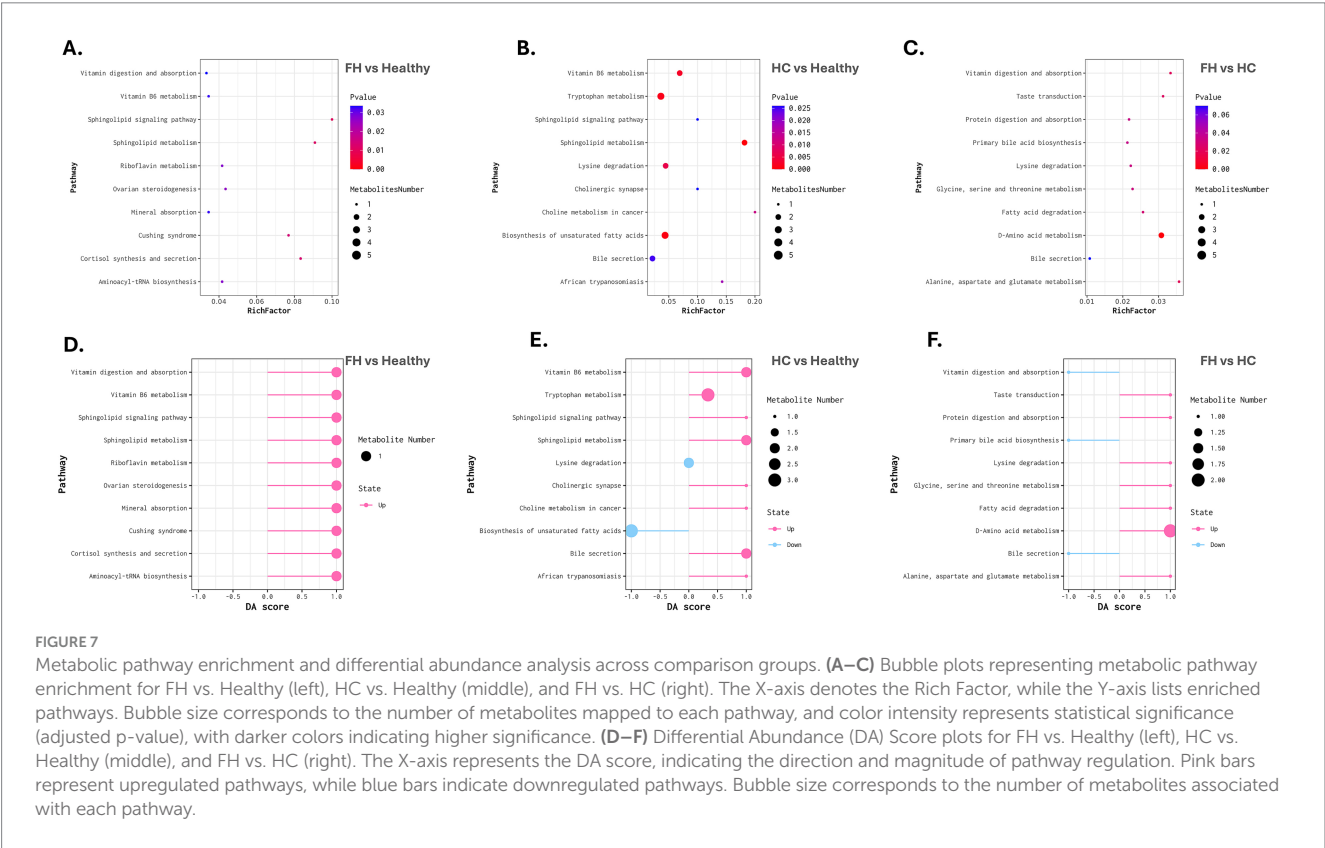
TABLE 1 KEGG pathway enrichment analysis across comparison groups.

Pathway ID	Pathway	Hit	Count	Adjusted <i>p</i> -value	KEGG Names	KEGG IDs
FH vs. Healthy						
map04071	Sphingolipid signaling pathway	1	10	0.0113	Sphinganine	C00836
map00600	Sphingolipid metabolism	1	11	0.0124	Sphinganine	C00836
map04927	Cortisol synthesis and secretion	1	12	0.0136	17alpha-Hydroxyprogesterone	C01176
map04934	Cushing syndrome	1	13	0.0147	17alpha-Hydroxyprogesterone	C01176
map04913	Ovarian steroidogenesis	1	23	0.0258	17alpha-Hydroxyprogesterone	C01176
map00740	Riboflavin metabolism	1	24	0.0269	Ribitol	C00474
map00970	Aminoacyl-tRNA biosynthesis	1	24	0.0269	L-Proline	C00148
map04978	Mineral absorption	1	29	0.0325	L-Proline	C00148
map00750	Vitamin B6 metabolism	1	29	0.0325	Pyridoxamine	C00534
map04977	Vitamin digestion and absorption	1	30	0.0336	Pyridoxamine	C00534
map05230	Central carbon metabolism in cancer	1	37	0.0412	L-Proline	C00148
map00310	Lysine degradation	1	45	0.0499	D-alpha-Hydroxyglutaric acid	C01087
HC vs. Healthy						
map00600	Sphingolipid metabolism	2	11	0.0004	Sphinganine; Phytosphingosine	C00836 C12144
map01040	Biosynthesis of unsaturated fatty acids	3	69	0.0008	Oleic acid; Linoleic acid; 11(Z),14(Z)-Eicosadienoic acid	C00712 C01595 C16525
map00380	Tryptophan metabolism	3	83	0.0013	L-Kynurenine; Indoleacetic acid;5-Hydroxyindoleacetic acid	C00328 C00954 C05635
map00750	Vitamin B6 metabolism	2	29	0.0026	4-Pyridoxic acid; Pyridoxamine	C00847 C00534
map00310	Lysine degradation	2	45	0.0062	D-alpha-Hydroxyglutaric acid; Glutaric acid	C01087 C00489
map05231	Choline metabolism in cancer	1	5	0.0131	Choline	C00114
map05143	African trypanosomiasis	1	7	0.0183	L-Kynurenine	C00328
map04976	Bile secretion	2	93	0.0249	Uric acid; Choline	C00366 C00114
map04071	Sphingolipid signaling pathway	1	10	0.0260	Sphinganine	C00836
map04725	Cholinergic synapse	1	10	0.0260	Choline	C00114
map00061	Fatty acid biosynthesis	1	12	0.0311	Oleic acid	C00712
map04931	Insulin resistance	1	12	0.0311	L-Acetylcarnitine	C02571
map02010	ABC transporters	2	118	0.0385	L-Histidine; Choline	C00135 C00114
map04723	Retrograde endocannabinoid signaling	1	15	0.0388	2-Arachidonoyl glycerol	C13856
map04714	Thermogenesis	1	17	0.0438	2-Arachidonoyl glycerol	C13856

(Continued)

TABLE 1 (Continued)

Pathway ID	Pathway	Hit	Count	Adjusted <i>p</i> -value	KEGG Names	KEGG IDs
FH vs. HC						
map00470	D-Amino acid metabolism	2	65	0.0011	D-Aspartic acid; D-Serine	C00402 C00740
map00250	Alanine, aspartate and glutamate metabolism	1	28	0.0216	D-Aspartic acid	C00402
map04977	Vitamin digestion and absorption	1	30	0.0231	Menadione	C05377
map04742	Taste transduction	1	32	0.0246	D-Serine	C00740
map00071	Fatty acid degradation	1	39	0.0299	Glutaric acid	C00489
map00260	Glycine, serine and threonine metabolism	1	44	0.0337	D-Serine	C00740
map00310	Lysine degradation	1	45	0.0344	Glutaric acid	C00489
map04974	Protein digestion and absorption	1	46	0.0352	4-Methylphenol	C01468
map00120	Primary bile acid biosynthesis	1	47	0.0359	Cholic acid	C00695



acid (52, 53). Concurrently, insulin resistance and impaired efficient fatty acid oxidation, evidenced by accumulation of β -oxidation intermediates like L-acetylcarnitine (54). These findings suggest that dietary interventions and metabolic therapies targeting fatty acid metabolism and insulin resistance may be especially beneficial for HC patients, more so than for those with FH.

HC patients also showed upregulation in the Bile secretion pathway with elevated levels of uric acid and choline, both of which are involved in cholesterol metabolism and inflammatory responses. Elevated uric acid levels have been linked with dyslipidemia and insulin resistance (55, 56). Choline is involved in lipoprotein synthesis and can reduce inflammatory markers (57, 58). These metabolites

likely reflect compensatory metabolic responses in HC and may act as HC-specific metabolic indicators.

Pathway enrichment analysis revealed that both FH and HC shared disruptions in sphingolipid metabolism and signaling. FH patients showed upregulation of sphinganine, while HC patients upregulated both sphinganine and phytosphingosine. These sphingolipids are involved in ceramide synthesis, which regulates cholesterol storage, efflux, and inflammation (59–61). In particular, sphinganine may contribute to disease progression and serve as a potential biomarker for cardiovascular risk in both HC and FH (12, 61–65). The broader sphingolipid remodeling in HC likely reflects the added effects of insulin resistance and inflammation. Additionally, lysine degradation was enriched by increased D- α -hydroxyglutaric acid in FH and HC, and decreased glutaric acid in HC only. Increased D- α -hydroxyglutaric acid, likely reflecting mitochondrial stress and disrupted energy metabolism (66). In HC only, glutaric acid was decreased, suggesting altered metabolic flux or increased downstream utilization toward lipid biosynthesis. These shifts may influence acetyl-CoA availability, a key precursor in cholesterol and fatty acid synthesis (67).

Vitamin B6 metabolism was also altered, with pyridoxamine upregulated in both FH and HC, likely reflecting oxidative stress and inflammation (68, 69). Interestingly, 4-pyridoxic acid was only elevated in HC, suggesting increased B6 turnover driven by metabolic stress, inflammation, and insulin resistance (70, 71).

Sphinganine, D- α -hydroxyglutaric acid, and pyridoxamine were altered in both FH and HC, suggesting shared disruptions in lipid metabolism and oxidative stress. In contrast, dysregulation of 4-pyridoxic acid, glutaric acid, and phytosphingosine was more pronounced in HC, likely driven by metabolic and lifestyle factors such as diet, obesity, and insulin resistance.

Our data have certain limitations that should be considered when interpreting the results. The relatively small sample size may limit the statistical power and the ability to capture the full spectrum of metabolic variation among individuals with FH and HC. In addition, several detected metabolic features remained unannotated, reflecting the current limitations of metabolite databases and highlighting opportunities for the discovery of novel biomarkers. While this study focused on untargeted profiling, the functional roles of the identified metabolites were not explored, which may serve as a valuable direction for future investigations to understand the mechanistic basis of the disease.

5 Conclusion

This first untargeted metabolomics study in Saudi patients comparing FH and HC revealed distinct metabolic profiles. FH showed altered bile acid and steroid hormone metabolism, marked by reduced cholic acid, elevated sphinganine, and 17 α -OHP. In contrast, HC exhibited lifestyle-related changes, including reduced UFAs, increased L-acetylcarnitine, uric acid, and choline, reflecting insulin resistance. Shared elevation of D- α -hydroxyglutaric acid and pyridoxamine suggests common mitochondrial stress, while glutaric acid, phytosphingosine, and 4-pyridoxic acid were more disrupted in HC. These metabolites may serve as biomarkers to distinguish FH from HC and support early diagnosis and personalized therapeutic strategies in different hypercholesterolemia forms.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The study was approved by the Biomedical Ethics and Research Committee of King Abdulaziz University, Jeddah, Saudi Arabia (reference number 220-22). All procedures were conducted in accordance with local legislation and institutional requirements. Written informed consent was obtained from all participants.

Author contributions

HA: Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. NS: Formal analysis, Supervision, Writing – original draft, Writing – review & editing. ZA: Data curation, Formal analysis, Supervision, Writing – review & editing. HD: Data curation, Writing – original draft. FA: Data curation, Writing – original draft. KA: Software, Writing – review & editing. AO: Formal analysis, Investigation, Writing – review & editing. RZ: Methodology, Writing – original draft. RH: Methodology, Writing – original draft. SE: Supervision, Writing – review & editing. BB: Supervision, Writing – original draft, Writing – review & editing. Software, Visualization. AM: Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmed.2025.1670282/full#supplementary-material>

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Glossary

FH - Familial Hypercholesterolemia

HC - Non-genetic Hypercholesterolemia

LDL-C - Low-Density Lipoprotein Cholesterol

LDLR - Low-Density Lipoprotein Receptor

PCSK9 - Proprotein Convertase Subtilisin/Kexin Type 9

LDLRAP1 - Low-Density Lipoprotein Receptor Adaptor Protein 1

17-OHP - 17 α -Hydroxyprogesterone

CVDs - Cardiovascular Diseases

GPC - Glycerophosphocholine

EP - Eppendorf

BGI - Beijing Genomics Institute

AGC - Automatic Gain Control

ESI - Electrospray Ionization

BMDB - BGI Metabolome Database

PQN - Probabilistic Quotient Normalization

QC-RLSC - Quality Control-based Robust LOESS Signal Correction

CV - Coefficient of Variation

KEGG - Kyoto Encyclopedia of Genes and Genomes

HMDB - Human Metabolome Database

DEM - Differentially Expressed Metabolite