Original Article



Integrated Analysis of Serum and Fecal Metabolites Reveals the Role of Bile Acid Metabolism in Drug-induced Liver Injury: Implications for Diagnostic and Prognostic Biomarkers

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Abstract

Background and Aims: Drug-induced liver injury (DILI) represents a prevalent adverse event associated with medication use. However, the exact mechanisms underlying DILI remain incompletely understood, and the lack of specific diagnostic and prognostic biomarkers poses significant challenges to the clinical diagnosis and treatment of this condition. Consequently, our study aimed to endeavor to identify serum and fecal metabolic biomarkers, enabling more accurate DILI diagnosis and improved prediction of chronic progression. Methods: Untargeted metabolomics analysis was performed on serum and fecal samples obtained from a cohort of 32 DILI patients (causality confirmed via the updated Roussel Uclaf Causality Assessment Method) and 36 healthy controls. Utilizing techniques such as partial least squares-discriminant analysis modeling and t-tests, we identified significantly differentially expressed metabolites and metabolite sets. Causality assessment was performed using the updated Roussel Uclaf Causality Assessment Method. Results: The findings from the analysis of serum and fecal metabolomics association pathways suggested that perturbations in bile acid metabolism might serve as potential mechanisms underlying the progression of DILI. Our study revealed 22 overlapping differential metabolites between serum and feces, displaying significant concentration differences between the DILI and healthy control groups. Notably, we identified chenodeoxycholic acid and deoxycholic acid as promising markers that not only distinguished DILI patients from healthy individuals but also exhibited predictive potential for DILI chronicity. Conclusions: The integrated analysis of serum and fecal metabolites uncovers the significant disruption of bile acid metabolites as a key contributing factor in the pathogenesis of DILI. Our study offers promising potential biomarkers for the diagnosis and prognosis of DILI,

paving the way for a novel perspective in the realm of DILI diagnosis and treatment.

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Introduction

Drug-induced liver injury (DILI) stands as the most prevalent adverse event and a prominent cause of acute liver fail-ure and transplantation.^{1–3} The incidence and impact of DILI have escalated in conjunction with the advent of new medications, increased life expectancy, polypharmacy among the elderly, and widespread utilization of herbal and dietary supplements.⁴ Although most DILI patients achieve full recovery upon discontinuation of the causative drug, approximately 20% experience a progressive course leading to chronicity, resulting in worse outcomes such as cirrhosis and, in severe cases, necessitating liver transplantation.⁵ Despite the growing attention toward DILI, its pathogenesis remains elusive, and the absence of specific biomarkers for accurate diagnosis and prognosis prediction poses a significant challenge.⁶ Hence, the exploration of molecular mechanisms and the identification of distinct diagnostic and prognostic biomarkers are imperative for effective DILI management, representing a critical area of focus in clinical practice. Given the liver's pivotal role in maintaining systemic homeostasis, numerous active metabolic processes within the organ are susceptible to perturbation caused by the deleterious effects of medications.⁷ Consequently, alterations in the metabolome hold the potential to elucidate the underlying mechanisms leading to DILI. Metabolomics emerges as a promising approach to identify metabolites that hold significant value in the quest for biomarkers and in unveiling the pathophysiological mechanisms associated with DILI.7

The liver possesses a distinctive vascular system that enables the direct reception of metabolites from the gastrointestinal tract and their subsequent processing. Given its anatomical position and specialized vasculature, the liver becomes a pivotal site for the metabolism of gut-derived

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metabolites, which play significant roles in the progression of DILI. Consequently, conducting a comparative analysis of metabolic changes in serum and stool samples between DILI patients and healthy individuals holds the potential to provide valuable insights into the intricate interplay of the gut-liver axis, aiding in the evaluation of potential etiological factors and identification of novel therapeutic targets for DILI patients. However, to date, there is a dearth of comprehensive metabolomics research systematically characterizing serum and fecal metabolites in the context of DILI. Therefore, the objective of this study was to undertake an in-depth serum and fecal metabolomics analysis of both DILI patients and healthy subjects, with the aim of uncovering previously unreported metabolic alterations specific to DILI patients.

Methods

Subject recruitment

This study involved the enrollment of hospitalized patients with DILI at the Fifth Medical Center of PLA General Hospital from July 2020 to June 2021. Ethical approval for the study was obtained from the Ethics Committee of the Fifth Medical Center of PLA General Hospital (Approval No. 2020050D). Prior to their participation in the study, all individuals involved, either personally or through their legal representatives, provided written informed consent. Demographic data, as well as laboratory test results, were collected from electronic medical records and questionnaires.

The study employed the following inclusion criteria: 1) participants between the ages of 18 and 69; 2) newly diagnosed with DILI, defined as diagnosis within the last three months; and 3) voluntary participation in the study, accompanied by the signing of the informed consent form upon enrollment.

The study employed the following exclusion criteria: 1) presence of concurrent liver injury due to various causes such as viral, autoimmune, alcoholic, genetic, metabolic, tumorous, or biliary diseases; and 2) individuals with autoimmune diseases, malignant tumors, or severe cardiac, pulmonary, or kidney failure.

The diagnostic criteria utilized for identifying DILI included the following:⁸ 1) recent occurrence of clinically significant abnormalities in liver biochemistry indices; 2) comprehensive assessment of medication and herbal/dietary supplement history within the 180-day period preceding presentation; and 3) exclusion of other potential causes of liver injury. Clinically significant abnormal liver biochemistry was determined by meeting any one of the following criteria:8 1) Serum concentrations of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) more than five times the upper normal limit, or alkaline phosphatase (ALP) concentrations more than twice the upper normal limit on at least two separate occasions spaced at least 24 h apart; 2) Total serum bilirubin (TBIL) concentrations more than 2.5 mg/dL, accompanied by increased serum AST, ALT, or ALP concentrations; or 3) International normalized ratio (INR) greater than 1.5, along with increased serum AST, ALT, or ALP concentrations. Based on the updated Roussel Uclaf Causality Assessment Method,⁹ cases with a score of 6 or more were identified as "probable" or "highly probable" DILI and included in this study. Chronic DILI was defined as the persistence of liver injury, histological evidence of significant fibrosis, or cirrhosis observed six months after the onset of DILI.8

Sample collection

After a minimum of 8 h of overnight fasting, stool and blood samples were collected in the morning. Blood samples were

drawn using coagulant tubes. The tubes were gently agitated and centrifuged at 3,000 rpm for 10 m at room temperature. The supernatant (serum) was carefully transferred into 1.5 mL cryogenic tubes and stored at -80° C. Freshly collected fecal specimens were immediately preserved at -80° C in a stool storage solution, pending further analysis.¹⁰

Untargeted metabolomics by ultrahigh-pressure liquid chromatography coupled with tandem mass spectrometry

In accordance with previous protocols,¹¹ the serum and fecal samples were transferred to EP tubes and mixed with precooled 80% methanol by thorough vortexing. The samples were then placed on ice for 5 m, followed by centrifugation at 15,000 g at 4°C for 20 m. To achieve a final concentration of 53% methanol, a portion of the resulting supernatant was diluted with LC/MS-grade water. The mixture was then transferred to a new Eppendorf tube and centrifuged again at 15,000 g at 4°C for 20 m. Finally, the supernatant was introduced into the LC-MS/MS system for analysis.

Statistical analysis

R software (version R-4.2.2) and Python (version 3.11.1) were used to perform all statistical analyses. A supervised partial least squares discriminant analysis (PLS-DA) model was applied to assess metabolic changes among different groups.¹² To evaluate the risk of overfitting, 200 permutation tests were conducted on the PLS-DA model. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was utilized for pathway enrichment analysis.¹³ The Pearson correlation coefficient was employed to evaluate the relationship between metabolites and liver function indices. Appropriate statistical tests were utilized to evaluate gualitative and quantitative differences among subgroups. These included the χ^2 test or Fisher's exact test for categorical variables, and the Student's t-test or Mann-Whitney U test for comparisons between two groups. Statistical significance was defined as a P-value less than 0.05.

Results

Baseline Characteristics

Our study included 32 DILI patients and 36 healthy controls (HC). The clinical characteristics of both groups are presented in Table 1. No significant differences were observed between the DILI and HC groups in terms of age, weight, sex, or body mass index (P > 0.05). However, the DILI group exhibited significantly elevated levels of ALT, AST, ALP, TBIL, total bile acid (TBA), gamma-glutamyl transpeptidase (GGT), and INR, while albumin (ALB) levels were significantly reduced compared to the HC group (P < 0.001) (Table 1). All suspected drugs causing DILI and the Roussel Uclaf Causality Assessment Method causality assessment items are listed in Supplementary Table 1.

Comparison of serum and fecal metabolic profiles in DILI patients and HC

A total of 1,589 serum metabolites and 2,298 fecal metabolites were detected using UPLC-MS. Unsupervised PCA demonstrated a distinct separation between DILI patients and HC in terms of serum and fecal metabolite profiles (Fig. 1A, B). Furthermore, the supervised PLS-DA model confirmed the significant differences in serum and fecal metabolic patterns (Fig. 1C, D). The reliability and validity of the PLS-DA model were further supported by a 200-permuta-

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Table 1.	Clinical	characteristics	of	68	subjects
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Clinical characteristics	DILI (n = 32)	Health control (n = 36)	P-value
Age, years, median (IQR)	50.0 (46.3-59.5)	52.0 (45.0-61.0)	0.662
Females, n (%)	19 (59.4)	20 (55.6)	0.751
Weight, kg, median (IQR)	60.0 (53.5-72.5)	63.0 (58.0-70.0)	0.389
BMI, kg/m ² , median (IQR)	21.6 (20.4–24.6)	22.8 (20.8–25.3)	0.685
Laboratory Examination			
ALT, U/L, median (IQR)	237.5 (97.0-506.8)	22.0 (12.0-26.8)	<0.001
AST, U/L, median (IQR)	168.5 (75.8-268.0)	26.0 (16.3-30.0)	<0.001
TBIL, μmol/L, median (IQR)	73.8 (32.7–124.9)	8.7 (5.8-13.0)	<0.001
ALP, U/L, median (IQR)	146.0 (92.8-224.0)	86.0 (77.3-113.5)	<0.001
GGT, U/L, median (IQR)	142.0 (82.0-221.3)	32.0 (18.0-40.5)	<0.001
ALB, g/L, median (IQR)	36.0 (32.0-39.0)	42.0 (39.3-46.0)	<0.001
TBA, µmol/L, median (IQR)	44.0 (11.0-97.8)	9.0 (4.3-16.3)	<0.001
LDH, U/L, median (IQR)	186.5 (158.8-205.8)	188.5 (147.0-242.0)	0.610
INR, median (IQR)	1.0 (1.0-1.1)	0.9 (0.9-1.0)	<0.001

DILI, drug-induced liver injury; IQR, interquartile distance; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total serum bilirubin; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; ALB, albumin; TBA, total bile acid; LDH, lactate dehydrogenase; INR, international normalized ratio.

tion test (Fig. 1E, F).

Applying a selection criterion of VIP value >1.0, fold change \geq 1.5 or \leq 0.67, and *P* < 0.05, we identified 225 differentially expressed metabolites (DEMs) in serum and 311 DEMs in feces between DILI patients and HC (Fig. 2A, B) (Supplementary Table 2). These metabolites encompassed various classes, with lipids and lipid-like molecules, organic acids and derivatives, and organoheterocyclic compounds being the major contributors to the observed differences (Fig. 3).

Correlation analysis of serum and fecal metabolites in DILI

Notably, we identified 22 overlapping DEMs between serum and feces (Fig. 4A), and their inter-correlations were visualized in a heatmap (Fig. 4C). KEGG pathway analysis revealed that the overlapping metabolites were primarily involved in primary bile acid biosynthesis (Fig. 4B). Additionally, metabolic perturbations in the serum of DILI patients compared to HC primarily affected the serotonergic synapse and cAMP signaling pathways, while in feces, they were associated with caffeine metabolism, cortisol synthesis and secretion, and Cushing's syndrome (Fig. 2C, D).

Interestingly, the inter-correlations among these 22 overlapping metabolites indicated that key metabolites involved in bile acid metabolism, such as glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid, glycocholic acid, chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and taurochenodeoxycholic acid, were increased in serum but decreased in feces (Fig. 5).

Metabolic biomarkers for diagnosis and prognosis prediction of DILI

DILI patients were followed for a period of six months, during which 17 chronicity events were documented. Patients were stratified into two cohorts: the non-chronic DILI group (n = 15) and the chronic DILI group (n = 17). No significant differences were observed in clinical laboratory parameters (AST, TBIL, ALP, GGT, TBA, INR) or demographic characteristics (sex, age, body mass index) between the two groups (Supplementary Fig. 1). To identify clinically actionable metabolite biomarkers, we prioritized metabolites that were differentially expressed in both serum and fecal samples. Using stringent selection criteria (fold change ≥ 1.5 or ≤ 0.67 , P < 0.05), six serum metabolites-CDCA, DCA, GDCA, androsterone glucuronide, testosterone sulfate, and 5-phenylvaleric acid-and three fecal metabolites (CDCA, DCA, and delta-tocopherol) were identified (Supplementary Table 3). Notably, CDCA and DCA exhibited consistent and significant alterations across both sample types (P < 0.01; Fig. 6), highlighting their dual utility as diagnostic and prognostic biomarkers for DILI. While CDCA and DCA levels significantly distinguished DILI patients from HC, their differences between hepatocellular injury-type DILI and cholestatic-type DILI subtypes did not reach statistical significance (Fig. 7). Furthermore, these metabolites showed no significant variations across DILI cases induced by different drug categories (Supplementary Fig. 2). This may reflect overlapping bile acid dysregulation mechanisms across DILI phenotypes.

Subsequently, we developed both a diagnostic model and a prognostic model based on these two metabolites. The diagnostic model demonstrated an area under the receiver operating characteristic of 0.836 (95% CI: 0.732–0.940, *P* < 0.001) in serum and 0.851 (95% CI: 0.756–0.945, *P* < 0.001) in feces (Fig. 8). The prognostic model exhibited an AUROC of 0.869 (95% CI: 0.745–0.923, *P* < 0.001) in serum and 0.837 (95% CI: 0.685–0.990, *P* = 0.001) in feces (Fig. 9).

Discussion

The incidence of DILI continues to rise, drawing increasing attention from experts in the field of liver diseases and emerging as a significant topic of discussion.¹⁴ Approximately 8% to 20% of DILI patients may develop chronic DILI or even progress to cirrhosis, necessitating lifelong treatment and, in severe cases, liver transplantation.^{15,16} Therefore, there is a pressing need to deepen our understanding of the underly-



Fig. 1. Visualization of serum and fecal metabolic profile differences between HC and DILI patients. (A, B) PCA score plots for the two groups; (C, D) PLS-DA score plots for the two groups; (E, F) Two hundred-time permutation test for model validation. HC group (n = 36, blue circles); DILI group (n = 32, red circles). HC, healthy controls; DILI, drug-induced liver injury; PCA, Principal Component Analysis; PLS-DA, Partial Least-Squares Discriminant Analysis.

ing pathogenesis and to identify novel biomarkers that can enhance the diagnosis of DILI and predict its progression to chronicity.

The pathogenesis of DILI is multifactorial and complex, with growing evidence highlighting the critical role of the gut-liver axis in its development and progression.¹⁷ The enterohepatic circulation of bile acids, in particular, is central to this axis and has been implicated in the pathophysiology of DILI. Disruptions in the gut microbiota and altered bile acid metabolism in the gut can directly affect liver function and contribute to hepatic injury.¹⁸ In our study, we observed significant alterations in both serum and fecal bile acid metabolites, reflecting dysregulation of the gut-liver axis in DILI patients. Bile acids are synthesized in the liver and secreted into the intestines, where they are modified by the gut microbiota into secondary bile acids. DILI can impair bile acid metabolism, resulting in enhanced intestinal reabsorption and disruption of enterohepatic circulation. This dysregulation may elevate serum bile acid levels while decreasing their fecal excretion, as observed in our findings. These changes not only contribute to liver injury but also perpetuate a cycle of inflammation and fibrosis as the liver attempts to restore homeostasis. Additionally, serum metabolomics revealed

disturbances in other pathways, such as the serotonergic synapse and cAMP signaling pathways, while fecal metabolomics identified alterations in caffeine metabolism, cortisol synthesis and secretion, and Cushing's syndrome-related pathways. These findings suggest that the distinct metabolomic phenotypes observed in serum and feces reflect the diverse pathophysiological changes occurring during DILI. Significantly, we identified two metabolites-deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA)--in both serum and feces that exhibited substantial differences in abundance among different disease states. Combinations of these two metabolites demonstrated predictive power for accurately classifying healthy individuals from those with DILI, as well as distinguishing between chronic and non-chronic forms of the disease, indicating their potential effectiveness for the diagnosis and prognosis of DILI.

The pathogenesis of DILI remains incompletely understood. Our study revealed significant alterations in bile acid metabolites, specifically GDCA, glycochenodeoxycholic acid, glycocholic acid, CDCA, DCA, and taurochenodeoxycholic acid, with elevated levels in serum and decreased levels in feces in DILI patients compared to healthy controls. This observation is particularly noteworthy, as high concentrations



Fig. 2. Differences in serum and fecal metabolites between the two groups. (A, B) Volcano plots of differential metabolites; (C, D) KEGG pathway analysis of differential metabolites. KEGG, Kyoto Encyclopedia of Genes and Genomes; TCA, tricarboxylic acid cycle.

of bile acids have been demonstrated to induce the production of reactive oxygen species and hepatocyte apoptosis, ultimately impairing liver function.¹⁹ Bile acid levels and the enterohepatic circulation significantly influence one another and are recognized as major contributors to DILI.²⁰ Enterohepatic circulation involves tightly regulated synthesis of bile acids, their secretion from the liver into the intestines, and subsequent reabsorption via the portal vein.²¹ Primary bile acids synthesized in the liver are transformed into secondary hydrophobic bile acids by specific gut microbes.²² DILI can disrupt this cycle and interfere with bile acid feedback mechanisms, resulting in enhanced intestinal reabsorption, elevated serum bile acid levels, increased biliary secretion, and reduced fecal excretion.^{20,23} Our findings are consistent with these mechanisms. We observed dysregulation of both primary and secondary bile acids along the gut–liver axis, highlighting unique metabolic alterations associated with DILI.

The hydrophobicity of bile acids plays a pivotal role in determining their cytotoxicity and protective effects.²⁴ Highly hydrophobic bile acids exhibit cytotoxic and pro-inflammatory properties.²⁵ In DILI, the intrahepatic accumulation and





retention of hydrophobic bile acids, such as CDCA and DCA, are well-recognized contributors to liver damage.^{25,26} Our study demonstrated that CDCA and DCA not only effectively distinguish DILI patients from healthy individuals but also possess prognostic value for predicting chronic DILI. These metabolites influence hepatic cholesterol and lipid metabolism as well as inflammatory responses during DILI progression.²⁶ Previous studies have shown that CDCA and DCA induce the expression of pro-inflammatory cytokines such as tumor necrosis factor-a and interleukin-1 β , which inhibit

the function and expression of farnesoid X receptor target genes, thereby amplifying inflammation.^{27–29} Additionally, CDCA and DCA can stimulate transforming growth factor- β 1 expression in hepatocytes, leading to activation of hepatic stellate cells.³⁰ These findings suggest that metabolic reprogramming of bile acids in DILI may intensify the inflammatory response and promote fibrosis via hepatic stellate cell activation, potentially driving chronicity. Inhibiting the accumulation of toxic bile acids in the liver may offer therapeutic benefits in chronic DILI, although further research is needed



Fig. 4. Integrated analysis of serum and fecal metabolites. (A) Venn diagram of overlapping differential metabolites; (B) KEGG pathway analysis of overlapping metabolites; (C) Correlation of overlapping serum and fecal metabolites. KEGG, Kyoto Encyclopedia of Genes and Genomes; HC, healthy controls; DILI, drug-induced liver injury.

to explore this potential.

This study represents the first attempt to integrate serum and fecal metabolomics in the context of DILI. Our findings reveal previously unreported alterations in bile acid levels in both the serum and fecal metabolome of DILI patients, underscoring the pivotal role of bile acid dysregulation in DILI pathogenesis. Although conventional liver biochemical markers (e.g., ALT, AST, TBIL) are highly sensitive for detecting hepatic injury, they lack specificity and cannot reliably distinguish DILI from other liver disorders. Notably, our results reveal that bile acid metabolites—CDCA and DCA—not only differ significantly between DILI patients and healthy controls but also correlate strongly with disease chronicity. These findings underscore the complementary diagnostic value of bile acid profiling in DILI management, offering two key advantages: (1) bile acid alterations may precede ALT/ AST elevations, enabling earlier clinical intervention; and (2) the association of CDCA and DCA with chronicity provides







Fig. 6. Box plots showing peak abundance of DCA and CDCA in different groups. (A) Peak abundance of DCA and CDCA in serum; (B) Peak abundance of DCA and CDCA in feces. *P < 0.05; **P < 0.01; ***P < 0.001; ns, P > 0.05. HC, healthy controls; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; NCD, non-chronic drug-induced liver injury; CD, chronic drug-induced liver injury.



Fig. 7. Box plots comparing peak abundance of DCA and CDCA between hepatocellular injury-type DILI (H-DILI) and cholestatic-type DILI (B-DILI). (A) Peak abundance of DCA and CDCA in serum; (B) Peak abundance of DCA and CDCA in feces. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, *P* > 0.05. HC, healthy controls; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; DILI, drug-induced liver injury.

predictive value for clinical outcomes—an area not addressed by conventional markers. This dual diagnostic and prognostic functionality positions bile acid metabolites as promising candidates for improving DILI assessment and bridging the gaps left by traditional liver tests.

However, several limitations must be acknowledged. First, the relatively small sample size of 32 DILI patients may limit the generalizability of our findings. Second, the absence of control groups with other liver diseases precludes definitive exclusion of non-specific liver injury effects on the observed metabolic changes. Published evidence suggests that bile acid profile disturbances in DILI may involve unique mechanisms, whereas other liver diseases, such as viral hepatitis or fatty liver disease, are more commonly associated with inflammatory or lipid metabolic dysregulation.^{31,32} Third, the absence of drug-treated controls who did not develop DILI limits our ability to differentiate metabolic changes caused by liver injury from those due to drug exposure alone.

Conclusions

Future studies incorporating such control groups would help clarify the causal role of drug administration in bile acid dysregulation and improve the specificity of biomarker identification. Although further validation is warranted, these metabolic signatures may provide complementary value for the early diagnosis and prognostic prediction of DILI. Future directions include multicenter, large-scale cohort studies to validate biomarker specificity, comparative metabolomic analyses across different liver diseases (e.g., viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease), and integrative multi-omics approaches to elucidate DILI-specific regulatory networks and enhance clinical applicability.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Design, writing of the manuscript (SY), data analysis (SW, PL), literature screening, data collection (HZ, JJ, TH), and re-

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Fig. 8. Diagnostic value of hub metabolites. (A) AUROC curve of hub metabolites in serum; (B) AUROC curve of hub metabolites in feces. DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; AUROC, Area under the Receiver Operating Characteristic; AUC, area under the curve; CI confidence interval.



Fig. 9. Prognostic value of hub metabolites. (A) AUROC curve of hub metabolites in serum; (B) AUROC curve of hub metabolites in feces. DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; AUROC, Area under the Receiver Operating Characteristic; AUC, area under the curve; CI confidence interval.

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vision of the manuscript (XD, RW). All authors have approved the final version and publication of the manuscript.

Ethical statement

We conducted a single-center, retrospective cohort study approved by the Ethics Committee of the Fifth Medical Center of PLA General Hospital (2020050D). All procedures were performed in accordance with the Declaration of Helsinki (as revised in 2024). All enrolled patients provided written informed consent.

Data sharing statement

The data supporting the findings of this study are available from the corresponding authors upon reasonable request.

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