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# Metabolomic analyses of faeces reveals malabsorption in cirrhotic patients

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ABSTRACT

*Background:* The study of faeces offers a unique opportunity to observe cooperation between the microbiome and the metabolism of mammalian hosts, an essential element in the study of the human metabolome. In the present study, a global metabolomics approach was used to identify metabolites differentially excreted in the faeces of cirrhotic patients compared to controls.
*Methods:* Seventeen cirrhotic patients and 24 healthy individuals were recruited. Faecal metabolites were detected through non-targeted reversed-phase ultra-performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry.
*Results:* A total of 9215 peaks were detected. Using unequal variance *t*-tests, 2393 peaks were observed with $P \leq 0.05$, approximately 74.0% of which were due to decreased faecal metabolite concentrations in liver cirrhosis vs. healthy controls. Integrating multivariate data analyses, we identified six major groups of metabolites. Relative levels of identified metabolites were as follows: strong increase in lysophosphatidylcholines, aromatic amino acids, fatty acids, and acylcarnitines, and a dramatic decrease in bile acids and bile pigments.
*Conclusion:* With severe hepatic injury in patients with liver cirrhosis, malabsorption occurs along with disorders of fatty acid metabolism, potentially due to changes in gut microflora.


## 1. Introduction

Liver cirrhosis is defined as the histological development of regenerative nodules surrounded by fibrous scar tissue in response to chronic liver injury of various etiologies, leading to progressive loss of liver function, and to altered liver metabolism. Cirrhosis and its associated complications are a major cause of morbidity and mortality worldwide [1]. Furthermore, 1–4% of cirrhotic patients with chronic hepatitis B or chronic hepatitis C develop primary liver cancer yearly [2]. Since liver cirrhosis is associated with serious sequelae, it is important for clinicians to better understand the pathophysiology of liver cirrhosis.

Metabolomics, a rapidly evolving tool in systems biology of small molecules, aims to identify untargeted potential biomarkers [3]. This technique offers great promise as an effective and non-invasive diagnostic method [4,5] and is a powerful approach for understanding the pathophysiology of diseases [6,7]. It can be applied to any biofluid, however tissue, blood and urine are the most frequently used specimens for exploring systematic alterations of metabolites in humans. Faecal samples are an obvious choice since they can be obtained easily and noninvasively, and can reflect the cooperation between microbiome–mammalian metabolism which is an essential element in the study of the human metabolome [8].

Discovering biomarkers of liver cirrhosis through a metabolomics approach is important for basic understanding of the mechanisms of liver damage, drug development, and clinical use (for example, diagnostic, prognostic biomarkers or therapeutic response). Previous metabolomic studies of liver diseases have found several biomarkers which provide new insights into the pathogenesis of hepatic diseases, including hepatic injury, oxidative stress and the abnormal metabolism of lipids and amino acids [5,9,10]. In the present study, we used ultra-performance liquid chromatography coupled with Q-TOF mass spectrometry (UPLC/Q-TOF MS) to analyze faecal samples collected from patients with liver cirrhosis in order to discover potential biomarkers, and gain new insights into the pathophysiology of cirrhosis.

## 2. Materials and methods

### 2.1. Study population

Written informed consent was obtained from all participants prior to initiating the trial and the study protocol conformed

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**Table 1**
Demographic and clinical characteristics of the study participants.

| Characteristic | Patient group ($n = 17$) | Healthy group ($n = 24$) | $p$-Value |
|---|---|---|---|
| Age (year) | 51.71 ± 10.02 | 47.13 ± 8.08 | 0.113 |
| Gender (male/female) | 11/6 | 17/7 | 0.678 |
| ALB, g/L (35–55) | 32.80 ± 3.77 | 46.56 ± 2.97 | <0.001 |
| ALT, U/L (3–50) | 48.88 ± 29.40 | 18.71 ± 7.74 | 0.001 |
| AST, U/L (3–40) | 83.89 ± 63.86 | 21.46 ± 6.26 | 0.001 |
| TB, μmol/L (1–22) | 44.94 ± 35.60 | 12.21 ± 4.10 | 0.002 |
| TBA, μmol/L (1–12) | 66.76 ± 61.11 | 2.93 ± 1.97 | 0.001 |
| PT, s (10.5–14.0) | 16.24 ± 3.46 | / | |
| Child–Pugh score | 8.06 ± 1.92 | / | |

Abbreviations: ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TB, total bilirubin; TBA, total bile acid; PT, prothrombin time. Results are reported as mean ± SD.

to the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by the Human Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University, China. Seventeen cirrhotic patients were recruited from patients who were admitted to the Department of Infectious Disease, The First Affiliated Hospital, College of Medicine, Zhejiang University during 2008–2009. These patients included nine patients with HBV-induced cirrhosis, five patients with hepatitis C virus-related cirrhosis, and three patients with alcoholic cirrhosis. A diagnosis of liver cirrhosis was confirmed using clinical signs, imaging findings, or evidence of esophagogastric varices. Patients with HIV co-infection or with hepatocellular carcinoma were excluded. The control group included 24 healthy individuals who came to the First Affiliated Hospital, College of Medicine, Zhejiang University for medical evaluation. They were confirmed to have normal liver function and no viral hepatitis, alcoholic or non-alcoholic fatty liver disease, or other diseases. Patients and volunteers did not undergo any gastrointestinal surgery or receive antibiotic treatment during the two months prior to the study. Subjects were advised to maintain their usual diet during the study period, and to avoid the intake of fermented foods. Demographic and clinical characteristics are shown in Table 1.

### 2.2. Sample collection and preparation

After obtaining informed consent, all stool samples were taken immediately following defecation, aliquoted, and stored at −80 °C until further use. Faecal water was extracted by taking a weighed sample of thawed stool, and mixing with methanol in a ratio of 3 ml/g. The mixture was homogenized using vortexing for 60 s, and then centrifuged at 10,000 rpm for 10 min. Supernatants were transferred to clean tubes, and then filtered through a membrane (0.22 μm pore size). The filtered faecal water was placed directly onto the column.

### 2.3. Chemicals

Acetonitrile and formic acid (HPLC grade) were purchased from Sigma–Aldrich (St. Louis, MO). Distilled water was purified "in-house" using a Milli-Q system (Millipore, Bedford, MA). Methanol, leucine-enkephalin, L-phenylalanine, L-tryptophan, L-tyrosine, chenodeoxycholic acid, lysophosphocholine (16:0), lysophosphocholine (18:2), lysophosphocholine (18:0), lysophosphocholine (18:1), linoleic acid and palmitoleic acid were purchased from Sigma–Aldrich. L-urobilin, L-urobilinogen and L-palmitoylcarnitine were purchased from J&K Chemical Ltd. (Beijing, China). 7-Ketolithocholic acid was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

### 2.4. Chromatography

A 2 μl aliquot was chromatographed on a (2.1 × 100 mm, ACQUITY™ 1.7 μm BEHC C18 column) (Waters, Milford, MA) maintained at 40 °C using a ACQUITYTM UPLC system (Waters, Milford, MA). The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The flow rate was 400 μl/min. A linear gradient was optimized as follows: 0–2 min, 3%–20% B; 2–5 min, 20%–30% B; 5–9 min, 30%–100% B; the composition was maintained at 100% B for 1 min, then for 9–12 min at equilibration with 3% B. The column eluent was directed to the mass spectrometer for analyses.

### 2.5. Mass spectrometry

Mass spectrometry was performed on a Waters Micromass Q-TOF Premier (Waters Micromass Technologies, Manchester, UK) operating in positive electrospray ionization (ESI) modes with "V-Optics". Capillary voltage and a cone voltage were set to 3000 and 35 V, respectively. Source temperature and desolvation temperature were maintained at 100 °C and 350 °C, respectively. Nitrogen was used as both the desolvation gas (600 L/h) and cone gas (50 L/h). The Q-TOF Premier MS acquisition rate was set to 0.3 s with a 0.1 s interscan delay. The scan range was from 50 to 1000 $m/z$. Data were collected in the centroid mode. All analyses were acquired using lock spray to ensure mass accuracy and reproducibility, and leucine-enkephalin was used as the lock mass ($m/z$ 556.2771) at a concentration of 200 ng/mL and a flow rate of 10 μL/min. Argon was used as the collision gas. The structures of faecal water biomarkers were elucidated using MS/MS fragmentation with collision energies ranging from 20 to 40 eV.

## 3. Data processing and statistical analyses

Data were analyzed using the MarkerLynx applications manager Version 4.1 (Waters, Manchester, UK). This application manager integrates peaks in the UPLC-Q-TOF MS data using ApexTrack peak detection. The track peak parameters were set as follows: peak width at 5% height 15 s, peak-to-peak baseline noise calculated automatically, minimum intensity 100, mass window 0.01 (Da), retention time window 0.2 min, noise elimination level 3, mass tolerance 0.01 (Da) with exclusion of de-isotopic data. Data were only used for 0–12 min.

The preprocessed data obtained by MarkerLynx were exported and analyzed using principal components analysis (PCA), and partial least squares discriminate analysis (PLS-DA) using SIMCA-P+ software (Umetrics). To globally analyze metabolomic differences between the liver cirrhosis group and the healthy group, unequal variance $t$-test analyses was performed with SPSS 16.0 for Windows (SPSS, Chicago, IL, USA).