Targeted Metabolomics Identifies Differential Serum and Liver Amino Acids Biomarkers in Rats with Alcoholic Liver Disease

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Summary To investigate changes in serum and hepatic levels of amino acids in ALD and to provide novel evidence and approaches for the prevention and treatment of ALD. Twenty specific pathogen-free SD male rats were divided into two groups, ten for the control group, and ten for the model group. Serum biochemical markers, including alanine aminotransferase, aspartate aminotransferase, laminin and hyaluronidase were measured. Histological analysis of liver tissues was performed. Serum and liver amino acids levels were quantitatively determined by ultra-high-performance liquid chromatography-tandem quadrupole mass spectrometry (UPLC-TQMS)-based targeted metabolomics. Compared with the normal group, ALD rats showed an obvious increase in the levels of β-alanine, alanine, serine, ornithine, tyrosine and the tyrosine ratio, while there was a decrease in arginine levels, the BTR ratio and Fischer’s ratio in serum. Additionally, ALD rats exhibited a significant increase in the levels of cysteine and putrescine, while there was a decrease in sarcosine, β-alanine, serine, proline, valine, threonine, ornithine, lysine, histidine, tyrosine, symmetric dimethylarginine, methionine, isoleucine and methionine-sulfoxide levels in liver tissues compared with the normal group. The serum and liver amino acids showed significant changes in ALD rats and can be considered as potential specific diagnostic biomarkers for ALD.

Key Words ALD, amino acid, targeted metabolomics, alanine, arginine

Alcoholic liver disease (ALD) is a chronic liver disease that is directly associated with long-term overdose alcohol consumption, including hepatic steatosis, alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma and liver failure (1).

Alcohol abuse has been as one of the top five risk factors for death and disability globally and results in 2.5 million deaths and 69.4 million annual disability adjusted life years (2). As a result, ALD has been a worldwide disease (2, 3) since its increasing incidence has made it a great threat to human health and social development. Approximately 60% to 90% of individuals who drink more than 60 g of alcohol per day have been shown to have hepatic steatosis, which means a high incidence of ALD because of the consumption of alcohol (4). ALD can adversely affect multiple organ systems such as gastrointestinal system, central nervous system, hematologic system, the cardiovascular system and renal system (5).

Many factors contribute to the development of ALD, such as oxidative stress, generation of reactive oxygen species (ROS) during alcohol metabolism, adipokines from visceral adipose tissue, and endotoxin derived from the gut, etc. (6). Of these, the most common and possible factors are oxidative stress and ROS, since the liver is the main site of alcohol metabolism. Alcohol dehydrogenase and cytochrome P-450 (CYP2E1) are the two main pathways of alcohol metabolism in the liver (7). Alcohol dehydrogenase converts alcohol to acetaldehyde, which is a hepatocyte cytosolic enzyme. Acetate is subsequently metabolized via the mitochondrial enzyme acetaldehyde dehydrogenase by acetaldehyde. It causes the reduction of nicotinamide adenine dinucleotide (NAD) to NADH. Via the inhibition of gluconeogenesis and fatty acid oxidation, the ratio of NAD/NADH is altered and may promote fatty liver development. CYP2E1 also converts alcohol to acetaldehyde. It is upregulated in chronic alcohol use, and free radicals are generated through the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to NADP. Hepatic macrophages are activated due to chronic alcohol abuse, and the activated macrophages produce tumor necrosis factor-α (TNF-α). TNF-α induces mitochondria to increase the production of ROS. Oxidative stress may promote hepatocyte necrosis and apoptosis. Free radicals initiate lipid peroxidation, which causes inflammation and fibrosis. These two pathways are considered the main ALD pathogenesis pathways. Although the pathogenesis of ALD has been widely investigated, the precise mechanisms remain to be elucidated. Thus, biomarkers for the early detection of ALD are urgently needed.
Metabolomics is a quantitative analytical technique, that measures all endogenous metabolites occurring in biosystems (cells, tissues and organisms) after excitations or interpretations from the external environment (8). It is a powerful technology that allows the assessment of global low-molecular-weight metabolites in biological systems and shows great potential in biomarker discovery (9). As is well known, the liver is the main location of alcoholic metabolism, and it is the most injured target organ (10). During the process of alcohol-induced liver injury, changes in hepatic metabolic pathways and metabolite levels may occur. By applying metabolomics to the research of ALD, the levels of differential metabolites and their effects on the development of ALD can be revealed.

Amino acids, as one of the most important nutrients in the metabolism of organisms, are the basic materials to make up proteins and are essential substances for organic nutrition (11). The main site of the catabolism of amino acids is the liver. In alcohol-induced liver disease, the level of the enzymes and some specific amino acids may show significant changes. In 2010, Mukherjee et al. (12) analyzed the patterns of changes in plasma amino-acid concentrations due to ALD. In 2015, Liang et al. (13) performed a metabolomics approach in a group of 206 ALD patients. A total of six differential urinary metabolites that contributed to ALD progress were identified, and more importantly, they discovered three of them with an accuracy of more than 95%. In 2016, Harada et al. (14) performed a population-based, cross-sectional study to identify potential biomarkers of alcohol intake and alcohol-induced liver injury by metabolomics profiling using capillary electrophoresis-mass spectrometry. Taken together, metabolomics has been applied and has provided a new approach to the study of ALD. However, the above studies did not systematically detect the amino acid levels using quantitative methods.

On the basis of these applications of metabolomics on ALD, our research on differential serum and liver amino acids in rats with ALD was performed based on ultra-high-performance liquid chromatography coupled with tandem quadrupole mass chromatography (UPLC-TQMS)-based quantitative targeted metabolomics analysis for detecting the pathogenesis of ALD and for searching for new approaches to prevent and treat ALD.

**MATERIALS AND METHODS**

**Animal experiments.** Twenty specific pathogen-free male SD rats were purchased from SLAC Laboratory Animal Center, Inc. (Shanghai, China). They were randomly classified into two groups, ten in the control group, and ten in the model group. A rat model of chronic ALD was established using a mixture (500 mL/L alcohol, 8 mL/kg per day; corn oil, 2 mL/kg per day; pyrazole, 24 mg/kg per day) once a day and intraperitoneal injections of 0.25 mL/kg of a 25% solution of CCl₄ in olive oil twice a week for 12 wk according to our previous study (15). After 12 wk, the rats were sacrificed. Blood samples were obtained, and serum was collected by centrifugation (12,000 rpm, 4°C, 10 min) and then stored at −80°C. Liver pieces were fixed in 10% neutral buffered formalin or snap frozen in liquid nitrogen for further analysis. The protocols of all animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Local Ethics Committee for Animal Research Studies at the Shanghai University of Traditional Chinese Medicine.

**Serum biochemical assay.** Serum biochemical assays, including measurements of alanine transaminase (ALT), aspartate transaminase (AST), laminin and hyaluronidase, were performed with an automatic biochemistry...
Liver histology and morphometry. Liver tissue fixed in formalin were then paraffin-embedded, and sectioned at 5 μm. For standard histology, liver sections were stained with Hematoxylin-Eosin (H&E). The stained sections were observed and photographed under a light microscope (with 200× magnification).

Serum and liver amino acid analysis using UPLC-TQMS based targeted metabolomics.

Preparation of internal standard: Isotopically labeled amino acids (16) were used as internal standards.

Table 1. Altered serum amino acid profiles in rats with ALD.

| AAs (µM) | Normal | Model | p-value |
|----------|--------|-------|---------|
| Creatinine | 40.975 (37.172–42.372) | 46.969 (43.244–52.738) | 0.274 |
| Glycine | 867.868 (814.128–933.445) | 1081.062 (1022.977–1138.628) | 0.147 |
| Sarcosine | 5.140 (4.759–5.926) | 4.88 (3.962–5.643) | 0.660 |
| β-Alanine | 8.828 (7.794–10.513) | 13.906 (11.872–16.305) | 0.039 |
| Alanine | 857.348 (845.415–883.310) | 989.483 (956.161–996.962) | 0.030 |
| Serine | 406.141 (397.772–416.530) | 548.262 (504.212–562.177) | 0.011 |
| Proline | 367.940 (330.322–389.071) | 369.197 (354.909–415.875) | 0.578 |
| Valine | 230.819 (224.673–241.771) | 230.093 (223.003–241.592) | 0.834 |
| Cysteine | 1.934 (1.259–1.804) | 1.474 (1.259–1.804) | 0.489 |
| Threonine | 455.176 (441.033–462.343) | 498.252 (475.911–514.262) | 0.069 |
| Taurine | 599.283 (556.882–646.830) | 643.545 (593.089–684.036) | 0.834 |
| OH-proline | 0.034 (0.029–0.044) | 0.053 (0.043–0.066) | 0.572 |
| Asparagine | 122.856 (118.437–129.463) | 124.440 (116.374–146.317) | 0.836 |
| Ornithine | 136.341 (134.844–137.824) | 613.048 (550.619–629.335) | 0.003 |
| Glutamic acid | 708.343 (678.051–759.150) | 709.055 (626.957–758.605) | 0.834 |
| Glutamine | 1752.128 (1708.983–1830.497) | 1777.516 (1716.758–1850.909) | 0.587 |
| Lysine | 910.093 (854.177–923.006) | 913.80 (854.177–923.006) | 0.089 |
| Histidine | 118.007 (93.569–114.917) | 118.380 (93.569–114.917) | 0.749 |
| Arginine | 474.466 (442.695–475.337) | 21.233 (7.322–78.512) | 0.002 |
| Citrulline | 134.356 (134.356–135.935) | 160.681 (143.379–175.820) | 0.117 |
| Tyrosine | 112.358 (106.455–118.547) | 121.672 (111.824–131.515) | 0.431 |
| Asymmetric dimethylarginine | 1.006 (0.944–1.275) | 1.529 (1.487–1.556) | 0.802 |
| Symmetric dimethylarginine | 0.357 (0.323–0.538) | 0.482 (0.389–0.712) | 0.247 |
| Methionine | 108.547 (96.695–110.221) | 117.618 (95.158–131.064) | 0.431 |
| Methionine-sulfoxide | 0.007 (0.006–0.014) | 0.008 (0.006–0.009) | 0.748 |
| Methionine-sulfoxide | 8.263 (6.935–8.601) | 6.704 (5.573–8.340) | 0.326 |
| Serotonin | 7.615 (6.551–8.491) | 7.301 (6.348–9.502) | 0.834 |
| Putrescine | 1.682 (1.609–1.804) | 2.729 (1.679–4.414) | 0.415 |
| Phenethylamine | 0.031 (0.020–0.057) | 0.063 (0.051–0.084) | 0.230 |
| Aspartic acid | 115.316 (100.544–133.551) | 100.604 (81.480–123.055) | 0.287 |
| Tryptophan ratio | 0.168 (0.106–0.174) | 0.154 (0.106–0.174) | 0.834 |
| Tyrosine ratio | 0.125 (0.120–0.125) | 0.175 (0.164–0.197) | 0.022 |
| BCAA/AAA ratio | 1.520 (1.425–1.615) | 1.344 (1.199–1.439) | 0.069 |
| BTR ratio | 5.469 (5.469–5.709) | 3.848 (3.207–4.215) | 0.036 |
| Fischer's ratio | 2.430 (2.247–2.561) | 1.917 (1.751–2.238) | 0.039 |
| Kynurenine/trypothan ratio | 0.019 (0.018–0.021) | 0.018 (0.014–0.027) | 1.000 |
| Serotonin/trypothan ratio | 0.051 (0.047–0.054) | 0.049 (0.043–0.067) | 0.674 |
| Glutamate/glutamine ratio | 0.406 (0.386–0.422) | 0.383 (0.346–0.409) | 0.574 |
| Phenylalanine/tyrosine ratio | 1.206 (1.106–1.219) | 0.924 (0.828–0.988) | 0.069 |

p value means when compared with Normal group.

Tryptophan ratio: tryptophan/(phenylalanine + tyrosine + valine + leucine + isoleucine); Tyrosine ratio: tyrosine/(phenylalanine + tryptophan + valine + leucine + isoleucine); BCAA/AAA ratio: (valine + leucine + isoleucine)/(tyrosine + phenylalanine + tryptophan); BTR (BCAAs/tyrosine ratio, BTR) ratio: (valine + leucine + isoleucine)/tyrosine; Fischer’s ratio: (valine + leucine + isoleucine)/(tyrosine + phenylalanine).

Sample preparation: Samples were prepared according to a previously reported protocol (17). Rat serum samples were thawed at 4°C, and then 10 μL of each serum sample was transferred into an eppendorf tube. Then, 10 μL of ddWater was added into each tube, and 5 μL of the internal standard mixture was added. Next, 40 μL of cold isopropanol (with 1% formic acid, v/v) was added to precipitate proteins and then was vortex mixed. All EP tubes were maintained at −20°C for
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20 min, and then, serum samples were centrifuged at 13,000 × g for 10 min. Then, 10 μL of the supernatant was transferred to a glass HPLC vial for derivatization. The details are shown in Supplemental Online Material, Fig. S1.

**Serum and liver amino acid analysis using UPLC-TQMS.**
UPLC-TQMS analysis was performed using an Acquity UPLC binary solvent manager, sampler manager, and column manager interfaced with a Xevo TQ-S tandem quadrupole mass spectrometer. MS/MS detection was via electrospray ionization (ESI) in positive ion mode using multiple reaction monitoring (MRM) for the quantification of each compound. Nitrogen was used as the desolvation gas, and argon was as the collision gas. The following source conditions were used: capillary voltage, 1.5 kV; source offset, 50 V; desolvation temperature, 600°C; source temperature, 150°C; desolvation gas flow, 1,000 L/h; cone gas flow, 150 L/h; nebulizer gas, 7.0 bar; and collision gas, 0.15 mL/min. The chromatographic separation used reversed-phase gradient chromatography on an HSS T3 2.1×150 mm, 1.8 μm column. The mobile phase was composed of 0.1% formic acid in water (v/v) (A) and 0.1% formic acid in acetonitrile (v/v) (B). The column temperature was maintained at 45°C, and linear gradient elution was performed at 0.6 mL/min starting at 4% B and held for 0.5 min before increasing to 10% over 2 min, then to 28% over 2.5 min, and finally increasing to 95% for 1 min, before returning to 4% B (1.3 min) for re-equilibration. The weak wash was 95:5 water/acetonitrile (v/v), and the strong wash was 100% isopropanol, respectively. The QC sample contained all the standards and internal standards. After the injection of a further double blank and a single blank, the analysis was started with injections of the calibration curve followed by a double blank injection. The QC standards were interspersed evenly throughout the study samples.

**Statistical analysis.** The raw LC-MS data were processed by the TargetLynx application package within MassLynx software. The raw data was smoothed, and peak integration was performed using ApexTrak algorithm. Multivariate models were constructed using partial least squares discriminant analysis (PLS-DA) using SIMCA-P 11.5 (Umetrics, Sweden). The models were validated using 7-fold cross-validation, and the valid and robust models were assessed using internal cross-validation (R2X, R2Y, Q2Y values) and permutation analysis. Mann Whitney U test was applied to determine if differences observed in concentrations between normal rats and model rats with ALD were statistically significant using SPSS 19.0 software (SPSS, Inc.). The
Liver injury appeared in ALD rats

The data were all described as the mean ± standard deviation (SD) and were analyzed by using nonparametric test. p < 0.05 was considered statistically significant.

RESULTS

Liver injury appeared in ALD rats

The rats in the normal group were more active than those in the model group. The model group rats only showed temporary excitement along with daily drinking, drunk and fallen asleep, and were even worse with the prolongation of modeling time. Compared with the normal group, serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) in the model group increased significantly (Fig. 1A). Combined with pathology changes observed with H&E stain (Fig. 1B), it could be determined that the model ALD rats were established successfully. The purpose of adding CCl₄ in the model is to make the ALD model more stable and to reduce the experimental period (18).
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Altered amino acid profiles in rats with ALD

Serum amino acid profiles. A total of 39 amino acids and 9 ratios in normal and ALD rats were determined in the present study. The detailed serum amino acid profiles in rats with ALD are shown in Table 1. It was shown that there were distinct differences between the normal and model groups in the 3D PLS-DA scores plot based on serum amino acids (R2X = 0.536, R2Y = 0.962, and Q2Y = 0.762) (Fig. 2A). Permutation analysis showed the reliability of the 3D PLS-DA model (Fig. 2B). The VIP values from PLS-DA score plots of differential amino acids in serum from ALD rats are shown in Table 2. Differential metabolites were obtained under the principle of VIP value > 1 from PLS-DA model and p < 0.05 from the nonparametric test. Serum β-alanine, alanine, serine, ornithine, tyrosine and tyrosine ratio were significantly higher in ALD (p < 0.05), while arginine, branched chain amino acids (BCAAs)/tyrosine ratio (BTR) ratio and Fischer’s ratio were lower in ALD (p < 0.05) compared with the normal control group. According to the VIP value, serum ornithine, arginine and serine (VIP > 1.3) may be the most important and possible differential metabolites between the two groups.

Liver amino acid profiles. The detailed liver amino acid profiles in rats with ALD are shown in Table 3. It was shown that there were distinct differences between the normal and model groups in the 3D PLS-DA scores plot based on liver amino acids, and the related parameters were R2X = 0.604, R2Y = 0.898, and Q2Y = 0.804 respectively (Fig. 3A). Permutation analysis showed the reliability of the 3D PLS-DA model (Fig. 3B). The VIP values from PLS-DA score plots of differential amino acids in liver from ALD rats are shown in Table 2, and differential metabolites were obtained under the principle of VIP value > 1 in the PLS-DA model and p < 0.05 from the nonparametric test. Liver cysteine and putrescine were significantly higher in ALD (p < 0.05), while sarcosine, β-alanine, serine, proline, valine, threonine, ornithine, lysine, histidine, tyrosine, symmetric dimethylarginine, methionine, isoleucine and methionine-sulfoxide were lower in ALD (p < 0.05) compared with the control group. According to the VIP value, urine methionine-sulfoxide and ornithine (VIP > 1.3) may be the most important and possible differential metabolites between the two groups.

DISCUSSION

ALD is a toxic liver disease caused by excessive or long-term drinking, and it is characterized by a rapid decline in liver function following a catastrophic insult to the liver (16). The liver plays a major role in amino acid metabolism and is central to the regulation of metabolic pathways. It is also responsible for the metabolism of hormones that affect protein, carbohydrate and lipid metabolism. Chronic and acute liver diseases can profoundly alter the nutritional status and amino acid metabolism of patients with ALD (19).

In the present study, we have demonstrated the changes of serum and liver levels of 39 amino acids and 9 amino acid ratios in normal rats and ALD rats which were quantitatively determined.

It is realized that the immunity mechanism is related to the pathogenesis and progression of ALD, while the amino acids constitute to the basic structural substances of the immune system. When alcohol is consumed in large quantities, the immune system is destroyed and the levels of amino acids show corresponding changes.

In the detection of serum amino acids, the levels of β-alanine, alanine, serine, ornithine, arginine, tyrosine, tyrosine ratio, BTR ratio and Fischer’s ratio were significantly different between the two groups. In the detection of liver amino acids, the levels of sarcosine, β-alanine, serine, proline, valine, cysteine, threonine, ornithine, lysine, histidine, tyrosine, methionine, isoleucine, symmetric dimethylarginine, methionine-sulfoxide and
putrescine were significantly different between the two groups (Fig. 4). Among these, four serum amino acids had levels that were contrary to the levels observed in liver tissue; these were β-alanine, serine, ornithine and tyrosine. The levels were significantly higher in serum, while lower in liver in ALD model group compared with normal control group. However, the detailed mechanism for this difference is unclear.

**Differential serum amino acid in ALD rats**

Amino acids are metabolized to provide energy and are also used to synthesize proteins, glucose, and/or other bioactive molecules (20). The serum level of β-alanine in ALD rats was significantly increased than that of normal rats, while the serum level of arginine was markedly decreased. Arginine can promote the ornithine cycle to convert more blood ammonia to urea, which can expedite the elimination and metabolism of blood ammonia (21). The decrease of arginine is a clear indicator of liver disease.

The tyrosine ratio is the ratio of serum tyrosine to valine, leucine, isoleucine and phenylalanine, which exhibited a slight increase in ALD rats. The BTR ratio is the ratio of serum valine, leucine and isoleucine to tyrosine, and it was significantly decreased in ALD rats compared to the control rats. Fischer’s ratio indicates the ratio of serum valine, leucine and isoleucine to tyrosine and phenylalanine, which showed a decrease in ALD rats compared to control rats (22). A low Fischer’s ratio has been shown associated with hepatic encephalopathy. All the above three ratios are considered good indicators of the severity of hepatic injury (23). In liver disease, the depletion of the BCAAs, including valine, leucine, and isoleucine, and increased concentrations of tyrosine, may affect the regulation of metabolic pathways. The perturbation of the urea cycle and a limited capacity for detoxification of ammonia with glutamine are associated with acute liver failure and, in particular, with hepatic encephalopathy, although the mechanism and prognostic significance remains unclear. As liver diseases progress, the imbalance of amino acids tends to become more marked, and aminograms are useful for assessing the prognosis of cirrhotic patients with or without hepatocellular carcinoma (HCC).

**Differential liver amino acid in ALD rats**

ALD rats showed a significant decrease in the levels of isoleucine and valine in liver than normal rats. These two BCAAs are among the nine essential amino acids. According to D’Antona et al. (24), the increase of BCAAs may prolong the lifespan of male mice, which was related to enhancing mitochondrial biogenesis and reducing ROS production by upregulating the expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α). Thus, it is supposed that the pathogenesis of ALD may be linked to ROS tissue damage. BCAAs were shown to be able to reduce hepatic apoptosis, promote hepatocyte regeneration and stimulate the production of hepatocyte growth factor. As a result, we found that BCAAs supplement may be a possible treatment approach to delay the progression of chronic liver injury. On the other hand, as two of the nine essential amino acids, they have stimulatory effects on hepatic protein synthesis and an inhibitory effect on proteolysis. The decrease of BCAAs in ALD also indicates that alcohol may cause a deficiency of nutrient intake (19). Zhao et al. (25) found that BCAAs exacerbated obesity-related hepatic glucose and lipid metabolic disorders through attenuating Akt2 signaling. Tedesco et al. (26) found that enriched in BCAAs reverted these molecular defects and mitochondrial dysfunction, suggesting that the mitochondrial integrity obtained with the amino acid supplementation could be mediated through a Sirt1-eNOS-mTOR pathway.

Moreover, amino acids are the major nitrogen source for glutamine synthesis in muscles. The synthesis of glutamine is activated during critical illnesses, such as cancer and trauma. However, the body demands for glutamine in certain physiologic conditions are enormous, and increased utilization of glutamine often
exceeds its synthesis, which finally results in its deficiency in plasma and muscles. The needs of BCAAs for synthesis of glutamine are connected with the breakdown of muscle proteins which results in muscle-protein wasting. It is reported that glutamine synthesis relies on glutamine synthetase in the cytosol of pericentral hepatocytes, where it ensures the clearance of ammonium and then controls blood ammonium concentration (27, 28). The decrease in the levels of liver amino acids may lead to the lack of apolipoprotein synthesis, so that triglyceride excretion may be disorder, which was the possible cause of ALD.

A significant decrease of sarcosine levels occurred in the livers of rats with ALD. Sarcosine is an acidic product of the liver that can provide energy for muscular cells (https://nootriment.com/sarcosine/).

In the present study, glycine was significantly lower, while cysteine was higher in the livers of ALD rats than in normal control rats. The exact reason for such phenomena is yet to be found. Cysteine and glycine are two amino acids that are required to synthesize hepatic glutathione (GSH). GSH is the most important antioxidant molecule in the human body, and it can be used to combat free radical damage (29). Alcohol can produce enormous free radicals during its metabolism in microsomes through cytochrome P4502E1 and P2E1 (30). Oxidative stress damage is the most important pathogenic mechanism of ALD. However, the experimental results did not support this pathogenic pathway, which remains to be evaluated in further studies.

Methionine was lower in the livers of ALD rats compared with normal rats, which may also be caused by oxidative stress damage. Alcohol and its metabolic production can produce oxidative free radicals that damage the activity of methionine synthetase (MA) and S-adenosyl methionine synthetase (MAT), which influences the synthesis of methionine and S-adenosyl methionine (SAM). Therefore, the disorder of liver methionine is a probable contributor to the pathogenesis of ALD, and the significant decrease in the level of methionine is a potential diagnostic biomarker for ALD.

Ornithine showed a marked decrease in the liver of ALD rats compared to the level in normal controls. It is one of the major amino acids in the ornithine cycle, which is mainly located in the liver. The liver can eliminate the toxicity of ammonia by synthesizing urea through the ornithine cycle. In that case, a decrease of ornithine means a decrease in the synthesis of urea, which may mean an impaired detoxifying function of the liver. Thus, a decrease in ornithine may be a possible diagnostic biomarker for ALD.

Putrescine showed a slight increase in the liver of ALD rats compared to that of normal rats. Putrescine is synthesized in small quantities by healthy living cells by the action of ornithine decarboxylase. The slight increase in putrescine could reflect prophase, which may contribute to the occurrence of ALD.

According to Prystupa et al. (19) in 2015, the amino acids were determined by automated ion-exchange chromatography, which is different from our UPLC-TQMS-based method. Tedesco et al. (26) also used UPLC-MS (AB Sciex, Milan, Italy) to detect amino acids in rodents, but they could only detect 15 amino acids simultaneously, including threonine, asparagine, tyrosine, serine, glycine, alanine, leucine, isoleucine, valine, proline, histidine, methionine, aspartic acid, glutamine, phenylalanine, glutamic acid, lysine, arginine, and tryptophan. However, our methods could simultaneously quantitatively detect the levels of 39 amino acids and 9 amino acid ratios using UPLC-TQMS based targeted metabolomics. Furthermore, our findings demonstrate that determination of certain amino acids which can be used to detect the diagnosis of ALD.

However, there are still several limitations in the present study. First, the number of the rats in each group is relatively small. Second, amino acid metabolomics analysis from patients with ALD would be better suited to illustrate the abnormal amino acid metabolisms in ALD than rat models. Furthermore, the detailed understanding of the mechanistic roles of specific amino acids in the pathogenesis of ALD needs to be evaluated in future research. In the future we will perform further research to understand the mechanistic roles of specific amino acids in the pathogenesis of ALD. We will further explore the related enzymes which involved in the metabolism of serum ornithine, arginine and serine, urine methionine-sulfoxide and ornithine, according to the information from KEGG pathway. As for ornithine, it is shown that four enzymes including ornithine racemase, amino acid racemase, d-ornithine 4,5-aminomutase subunit beta, d-arginase are involved in the synthesis of ornithine, another three enzymes including alanine transaminase, d-ornithine/d-lysine decarboxylase, d-amino-acid oxidase are involved in the metabolism of ornithine. We can further perform RNA sequence to find differential molecules, and then use modern technologies such as CRISPR/CAS9, targeted metabolomics, adeno virus transfection through animal and cell models by focusing on related molecules which are involved in those differential metabolites to further explore the deep mechanisms.

In conclusion, the application of targeted UPLC-MS/MS analysis provides a useful approach to study the key effect of amino acids in the pathogenesis of ALD. Amino acids can be considered as specific diagnostic biomarkers for ALD. The metabolic patterns of amino acids can provide theoretical evidence for pathogenic pathways in ALD and can offer new ideas and targets for the prevention and treatment of ALD.

Authorship

T.W. initiated and supervised the whole manuscript; C.Z.S., L.W. and K.J.Z. performed the experiment; K.J.Z. and T.W. analyzed the data; M.M.S. and Y.F.L. participated in the data collection; C.Z.S. and L.W. wrote the manuscript; K.J.Z. and T.W. revised the manuscript. All authors have read and approved the final manuscript. C.Z.S and L.W. are co-first authors. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for
publication.

Disclosure of state of COI
The authors have no conflicts of interest to declare.

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Supporting information
Supplemental online material is available on J-STAGE.

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