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Research article

Serum Amino Acids Profiling In NAFLD Mice

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ABSTRACT: **Objectives:** To detect the variation of serum amino acids in control and nonalcoholic fatty liver disease (NAFLD) mice by PITC pre-column derivatization HPLC, and further explore the potential biomarkers associated with the development of NAFLD using metabolism profiling analysis. **Methods:** Male C57BL/6J mice were fed with high fat and high fructose diet, after 8 weeks, serum samples of control and model mice were collected. Serum biochemical parameter and liver histopathology were examined, and then metabolic profile was further analyzed using PITC pre-column derivatization HPLC. Result: Levels of serum and hepatic biochemical parameter and liver histopathology verified the NAFLD model successfully. The metabolism profile of NAFLD model mice was significantly different from that of the control mice. **Conclusion:** Pattern recognition method demonstrated that there were significant differences in the five amino acids markers (glutamic acid, glycine, methionine, isoleucine, valine) between the control and NAFLD mice. Amino acids metabolism profile combined with pattern recognition technology may reflect to a certain extent the metabolism changes of NAFLD mice. It also provided the potential scientific and clinic value for further study of NAFLD.

KEYWORDS: HPLC; amino acid; nonalcoholic fatty liver disease.

Statement of Originality of work: The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and that each author believes that the manuscript represents honest and original work.

INTRODUCTION

Amino acid is the constituent units of protein and the material basis of life activity. It is involved in the synthesis of bioactive substances (hormones, enzymes, vitamins, hemoglobin, nucleotide, glutathione, nitric oxide, neurotransmitter, coenzyme, etc coenzyme, etc). Most of the amino acids which were digested and absorbed are decomposed in liver. Under the action of the liver transaminase, amino acid can be converted into glucose, lipid, non-essential amino acid, protein and so on. Studies found that levels of serum amino acids would change after liver cell

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damage[1-3]. The amino acid imbalance theory thought that serum amino acid metabolism developed serious disorder after liver function obstacle[4]. Nonalcoholic fatty liver disease is a common chronic liver disease. The incidence rate is increasing year by year and it has become the biggest liver disease[5,6]. The pathological progress ranging from simple fatty liver to nonalcoholic steato hepatitis (NASH), and liver fibrosis, may eventually turn into cirrhosis and hepatocellular carcinoma. And clinic pathologic syndromes are mainly characterized by fat storage and steatosis in the liver parenchyma cells, but the pathogenesis is not yet clear[7]. Lots of studies reported that liver disease was associated with amino acid metabolism, but few studies to explore relationship between NAFLD and amino acid metabolism. Our study found that there were significant differences in amino acids between control and NAFLD mice using HPLC method combined with pattern recognition technology. It will provide the potential scientific value for the in-depth study, and clinical values for diagnosis and treatment of NAFLD.

MATERIAL AND METHODS

CHEMICALS AND REAGENTS

Amino acid standard substance: aspartate (Asp), glutamic acid (Glu), serine (Ser), glycine (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), tyrosine (Tyr), valine (val), methionine (Met), isoleucine (ile), leucine (leu), phenylalanine (Phe), tryptophan (Trp), lysine (Lys) and internal standard (leucine, Nle). HPLC grade acetonitrile, sodium acetate and methanol were obtained from Merck, Germany. Analytical grade such as phenyl isothiocyanate and hexyl hydride were purchased from commercial sources. Ultra-pure water was obtained from a Sartorius Arium 611VF system.

Instrumentation and chromatographic conditions

HPLC separations were performed on a Shimadzu LC-2010C system (Japan) equipped with a quaternary pump, temperature-controlled column compartment, an automatic sampler injector, a SPD-M10AVP detector, an online degasser and a data system (Shimadzu' LC solution). Electronic balance (ten thousandth units, Shanghai precision scientific instrument company). Chromatographic separation was achieved by using a phenomenex C18column (250mm×4.6mm, 5μm). The system was run in gradient elute with mobile phase consisting of methanol: water: acetonitrile (A, 20:60:20, v/v) and sodium acetate buffer: acetonitrile (B, 93:7, v/v). Elution ratio was shown in Table 1. The eluent was monitored at 430nm and at a flow rate of 1 mL/min. The injection volume was 20 μL.

Table 1. Mobile phase ratio

Time (min)	A (%)	B (%)
0	0	100
5	0	100
6	4.7	95.3
19	4.7	95.3
22	30	70
32	28	72
37	40	60
40	45	55
60	80	20
70	80	20

ANIMALS AND DIET

Male C57BL/6J mice weighing 18–22g were purchased from Guangdong Medical Laboratory Animal Center (GDMLAC) (NO. 44007200005269). All experiments involving mice conformed to the guidelines for animal care and used of the People's Republic of China and were approved by the animal ethics committee of the Chinese Academy of Medical Science (Beijing, People's Republic of China). Normal diet and High-fat diet (HFD, 71.9% normal diet, 18% lard oil, 10% cane sugar, 0.1% tapazole) were purchased from GDMLAC. All mice were housed alone in standard cages for one week. The animals kept at a controlled temperature of 24±2 °C, 55%–60% relative humidity and 12h light/dark cycles. Mice were free access to food and water ad libitum and weighed twice a week throughout the experiment. According to the random number table, the animals were randomly divided into two groups: (1) normal chow diet (Control group, n=8) (2) high fat diet plus 10% high fructose liquid (HFDL) (Model group, n=8) for 8 weeks to induce NAFLD. On 56th day, body weight and body length were measured, blood was collected from the retroorbital plexus by using microcapillary tubes after overnight fasting, and then centrifuged at 3000 rpm for 15 min. The animals were sacrificed

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by cervical dislocation, and tissues were rapidly removed. Among these tissues, liver were weighed and a portion of the liver was fixed in 4% paraformaldehyde for histopathological examination, while the remaining tissue immediately frozen in liquid nitrogen for further analysis.

PREPARATION OF THE CALIBRATION STANDARDS

Stock solutions of amino acid standard (100 μg/mL) and Nle (internal standard, 100 μg/mL) in 0.1 mol/L HCl were prepared and stored at 4℃ .Concentrations of standard solution (100, 50, 25, 12.5, 6.25, 3.125, 1.56 μg/mL) was prepared.

SAMPLE PREPARATION

All frozen samples were thawed at room temperature prior to process. Then, 10 μL of IS solution (100 μg/mL) was added to 100 μL serum sample. After mixing, 400 μLof acetonitrile was added. The mixture was vortexed for 30 s and standing for 10 min followed by centrifugation at 12,000 rpm/min for 10 min. The supernatant was carefully separated to a glass tube and evaporated to dryness at 40 °C under the nitrogen environment. After that, the residue was redissolved in 100 μL water and centrifugation at 10,000 rpm/min for 5 min. And then a 20 μL of the supernatant was injected into analytical column.

AMINO ACID DERIVATIZATION REACTION

50 μL of triethylamine acetonitrile solution and 50 μL of isothiocyanate phenyl acetonitrile solution (0.1 mmol/L) were added to 100 μL of amino acid standard solution, mixing. After standing for 10 min, 200 μL of hexyl hydride was added.
The mixture was vortexed and then centrifugated at 10,000 rpm/min for 5 min. The substrate was carefully collected and the extraction step was repeated twice. After centrifugation at 10,000 rpm/min for 5 min, then a 20 μL of the supernatant was injected into analytical column.

MULTIVARIATE DATA ANALYSIS AND BIOLOGICAL MARKERS IDENTIFICATION

Principal component analysis (PCA) and partial least squares – discriminant analysis (PLS-DA) were built by SIMCA-P + 11.5 software (Umetrics, Umea, Sweden).The validity of the model was verified by performing permutation tests. Variable importance value (VIP, > 1) from PLS-DA and *p*-value (*< 0.05*) of independent t - test with Welch's correction determined the discriminant the variables between different classes.

DATA AND STATISTICAL ANALYSIS

Data's were expressed as mean ± SEM, calculations and the graphs were generated using Graph-Pad Prism 5 (Graph-Pad Software, La Jolla, CA). Statistical comparisons were performed using the one-way and two-way ANOVA of variance followed by the Tukey–Kramer multiple-comparisons test where appropriate. The results were considered significantly different at $p < 0.05$.

RESULTS

Body weight, liver index and BMI in NAFLD mice
After C57BL/6J mice were fed the HFDL for 8 weeks, mice were exhibited a marked increase ($p < 0.01$)in body weight, liver index (liver weight/body weight×100%) and body mass index (BMI, body weight/body height 2×100%) compared with the control (Table 2).The result indicated NAFLD were associated with obesity and liver weight.

Table 2. Changes of body weight, liver index and BMI in two group mice.

Group	Body weight (g)	BMI (%)	Liver index (%)
Control	19.53±0.89	64.12±0.02	3.89±0.04
Model	23.76±0.63*	78.25±0.04*	5.18±0.13**

Data were expressed as means ± SEM (n=8), $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control.

SERUM LIPID AND FBG IN NAFLD MICE

In the study, mice fed by HFDL chow have higher levels of fasting blood glucose (FBG), TG, CHOL, and LDL-C than the control group, but levels of HDL-C has no significant different in two groups (Table 3).The result indicated NAFLD mice increased level of FBG and serum lipid.

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Table 3.Changes of blood glucose, serum lipid in two group mice.

Group	FBG (mmol/L)	TG (mmol/L)	CHOL (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
Control	5.05±0.76	0.85±0.07	2.10±0.12	0.16±0.01	1.41±0.05
Model	9.87±0.58***	1.11±0.13*	4.24±0.26***	0.63±0.04**	1.11±0.03

Data were expressed as means ± SEM (n=8), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with control.

TG AND CHOL IN LIVER OF NAFLD MICE

We detected the level of TG and CHOL in liver of control group and model group mice. The result showed that TG and CHOL obviously accumulated in liver of mice treated with HFDL comparing with control group. (Fig. 1)

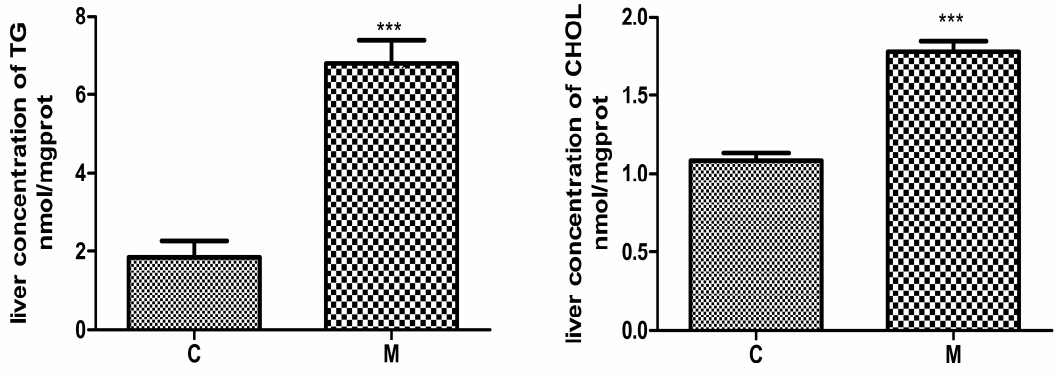

Figure 1. The concentration of TG and CHOL in mice liver. C: control group; M: model group. Data were expressed as means ± SEM (n=8). *** $p < 0.001$, compared with control group.

SERUM TRANSAMINASE IN NAFLD MICE

The levels of serum transaminase are the major sensitive indicator of hepatic injury. The result found that compared with the control group, progression of NAFLD obviously increased the levels of both serum alanine amino transaminase (ALT) and aspartate amino transferase (AST) (Table 4).

Table 4 .Changes of serum transaminase in two group mice.

Group	ALT (mmol/L)	AST (mmol/L)
Control	33.11±1.32	140.50±6.59
Model	54.33±2.42**	236.33±14.90**

Data were expressed as means ± SEM (n=8), *$p < 0.05$, **$p < 0.01$, compared with control.

HEPATIC HISTOLOGY IN NAFLD MICE

Liver histological analysis with Hematoxylin and eosin (H&E) staining is NAFLD diagnostic gold standard. According to results of our study, the development of hepatic steatosis was performed. Liver sections from the control group had normal morphological appearance. Liver sections from model group appeared a large number of diffuse fat vacuoles, higher degree of macrovesicular steatosis, hepatocellular ballooning and obvious inflammatory sites (Fig. 2).

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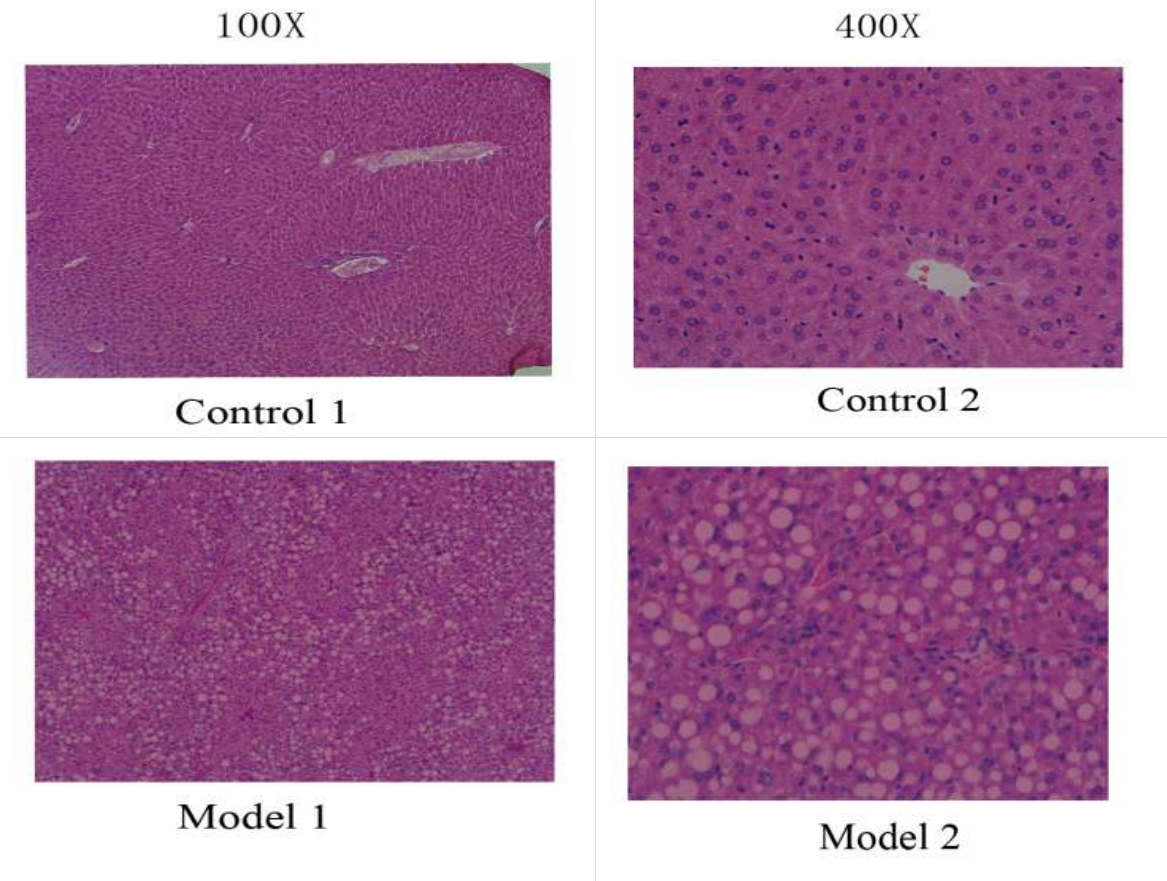
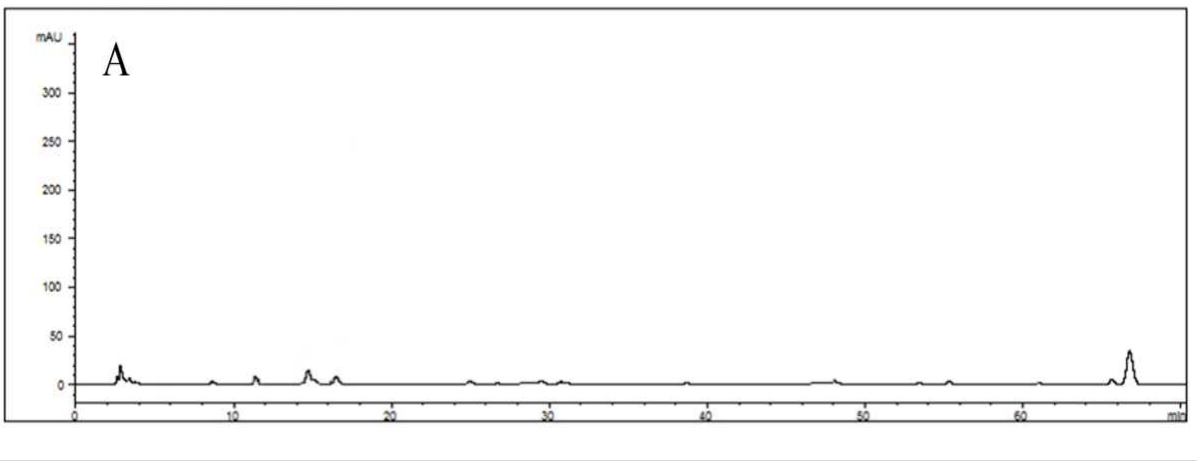


Figure 2. Liver tissue HE stain of two group mice left: 100X, right: 400X）

ANALYSE OF SERUM AMINO ACID METABOLISM BY HPLC

In this study, serum amino acids were well separated by gradient elution in HPLC chromatogram(Fig.3 A-C).The levels of Glu、Val、Ile、Leu were reduced significantly in the model group compared with the levels in the control group mice. But levels of Gly、Thr、Ala、Pro、Met、Trp and Lys were elevated significantly in model group compared to the control group (Table 5 and Fig. 4).



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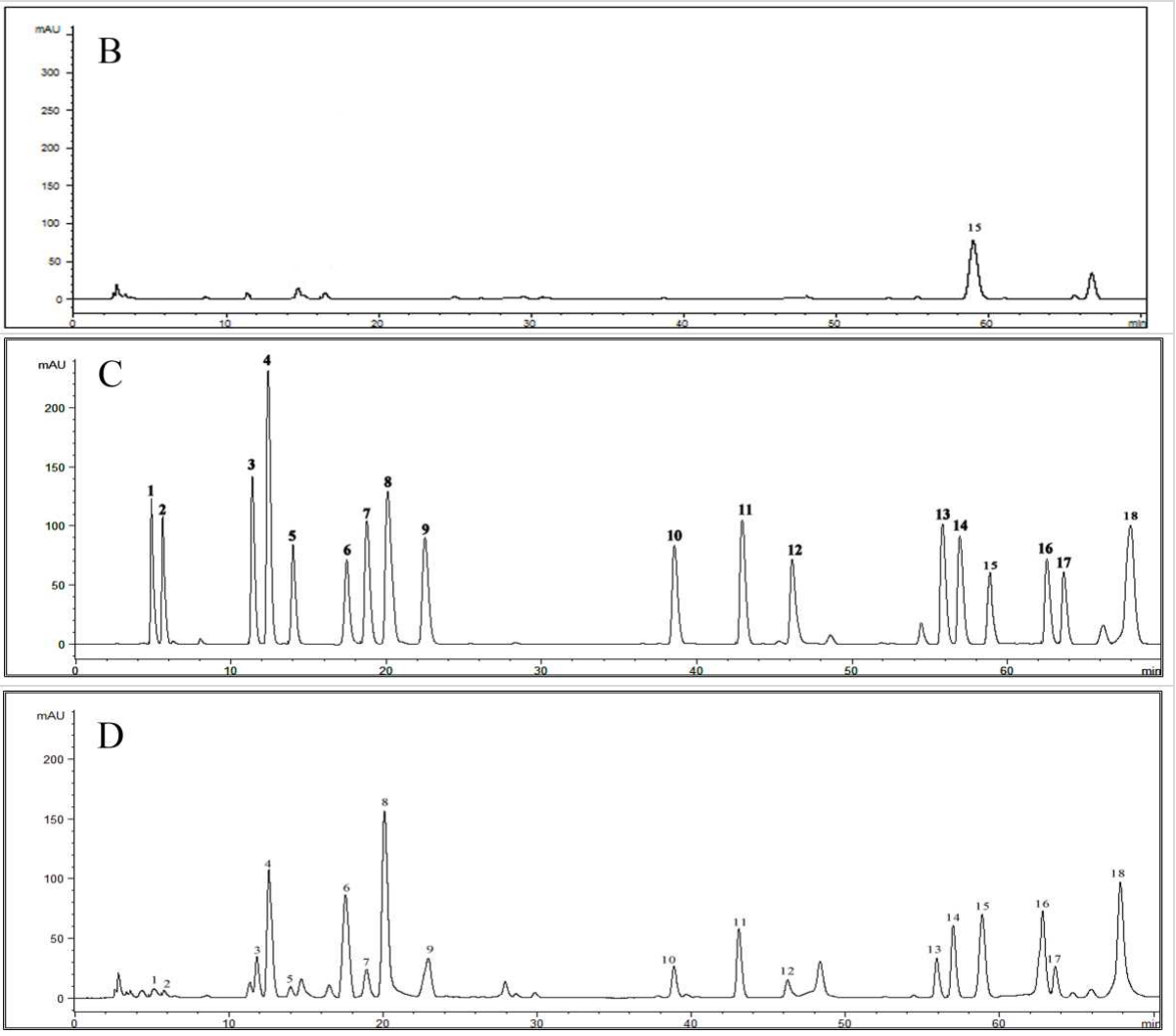

Figure 3. Analyse of amino serum acid metabolism by HPLC. Representative HPLC chromatograms of (A) Blank serum; (B) Internal standard; （C） 17 kinds of amino acid standard; (D) Amino acids sample for C57BL/6Jmice.

Table 5. Concentration of amino acids in two groups.

Number	amino acid	Contol	Model
1	Asp	4.32±0.32	8.02±0.59
2	Glu	25.97±1.41	15.36±1.28***
3	Ser	4.70±0.59	11.92±0.72
4	Gly	26.83±2.39	14.65±1.01***
5	His	2.58±0.15	7.25±0.34
6	Arg	14.62±1.76	9.86±0.68
7	Thr	9.02±1.76	16.42±1.07*
8	Ala	24.76±2.61	43.9±3.98***
9	Pro	8.44±1.58	19.17±2.77***

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10	Tyr	6.32±0.49	11.00±0.75
11	Val	29.90±1.51	20.33±1.38**
12	Met	7.06±0.55	16.14±1.35**
13	Ile	15.86±0.55	6.71±0.97**
14	Leu	28.80±1.25	19.96±2.01**
15	Phe	12.34±2.41	17.89±1.53
16	Trp	10.90±1.47	18.63±2.15*
17	Lys	25.38±2.81	45.54±3.63***

Data were expressed as means ± SEM (n=8), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

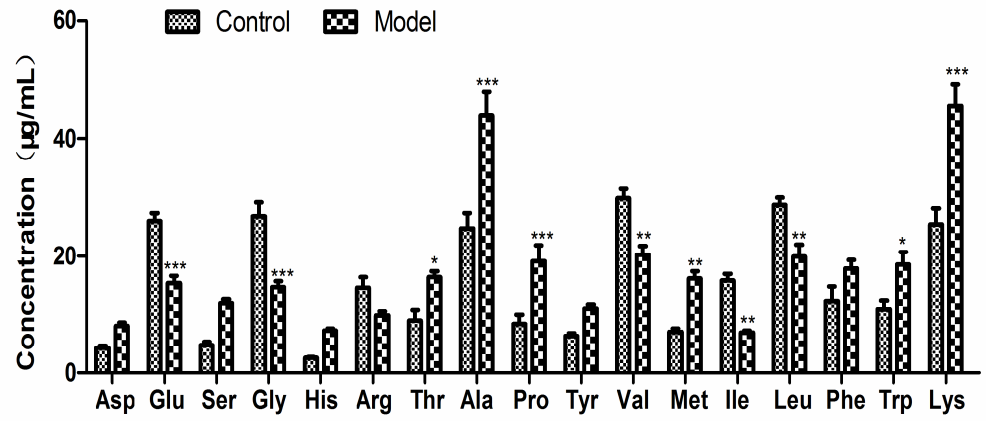
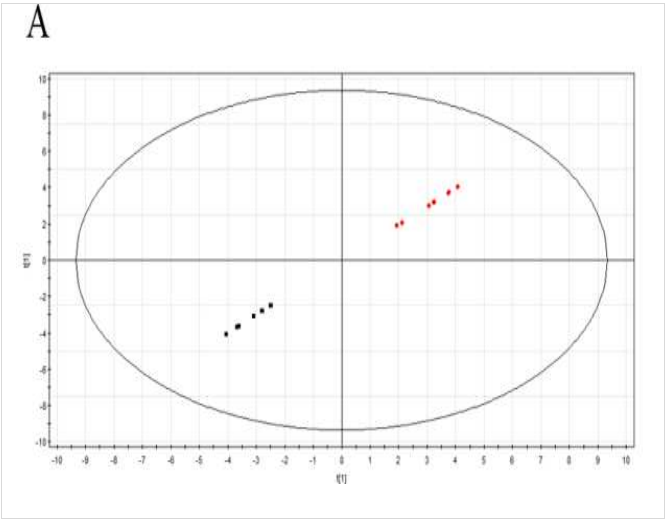


Figure 4. Changes of amino acids levels in two groups. Data were expressed as means ± SEM (n=8), * $p < 0.05$, ** $p < 0.01$, *** p < 0.001, compared with the control group.

ANALYSIS OF AMINO ACIDS METABOLISM SPECTRAL AND SCREENING OF BIOLOGICAL MARKERS

In our study, unsupervised PCA analysis showed a good separation between the two groups (R^2X=0.641 and Q^2=0.5435) (Fig.5 A). The separation became clearer by using supervised PLS-DA analysis (R^2X=0.743, R^2Y=0.981 and Q^2=0.961), indicating that there is obvious difference in amino acids metabolism spectra between the two groups (Fig.5 B). Permutation test further confirmed validity and reliability of PLS-DA model (Fig.5C).According to a VIP value > 1 and a*p*value < *0.05* of *t*-test, we screened some metabolites with significant difference as biological markers of NAFLD, and would contribute to further study NAFLD (Table.6).



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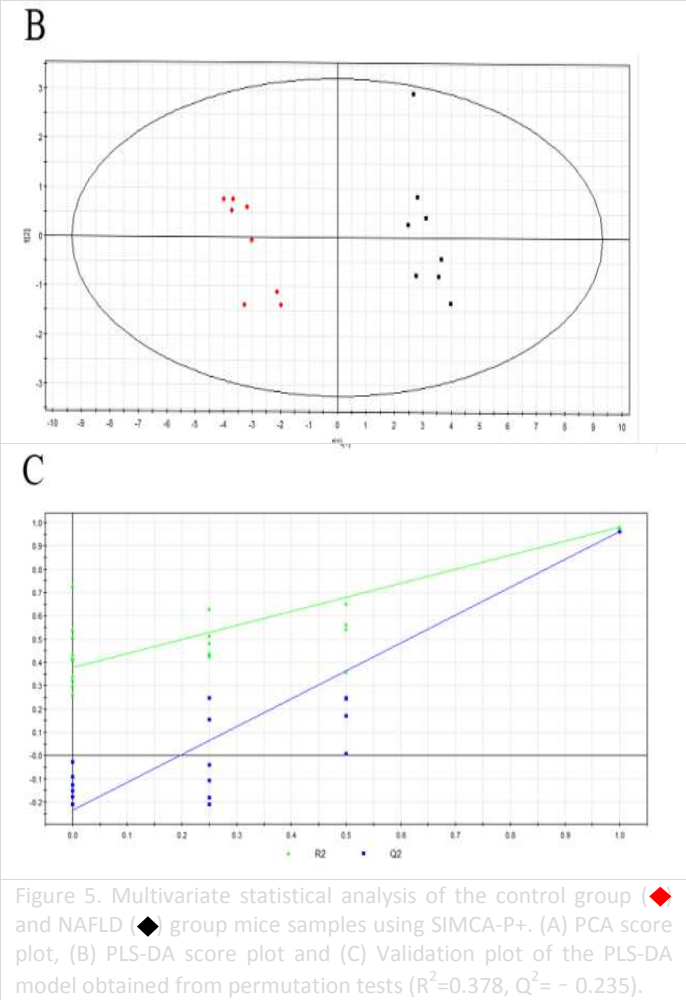

Figure 5. Multivariate statistical analysis of the control group (◆) and NAFLD (◆) group mice samples using SIMCA-P+. (A) PCA score plot, (B) PLS-DA score plot and (C) Validation plot of the PLS-DA model obtained from permutation tests (R^2=0.378, Q^2= – 0.235).

Table 6. Marker metabolites found in PLS-DA models of HPLC-based metabolic profiling..

Amino acids	Variations versus the control group	Metabolism pathways
Glutamic acid	↓	Glutathione metabolism
Glycine	↓	
Methionine	↓	
Isoleucine	↓	Branched-chain amino acid (BCAA) metabolism
Valine	↓	

The arrows ↓ indicated decrease of levels in the NAFLD group compared with the control group, respectively.

DISCUSSION AND CONCLUSION

NAFLD is the most common liver disease in the world, and the secular prevalence trend of NAFLD is both alarming and worrying[8]. More and more studies reported that NAFLD was usually associated with the metabolic syndrome, such as obesity, diabetes mellitus, insulin resistance (IR), hyperglycemia and hyperlipidemia[9-11]. Though the pathogenesis is not yet clear, researchers found that it is accompanied by abnormities of glucose metabolism, lipid metabolism, energy metabolism and amino acids metabolism in NAFLD. Metabolomics is a rapidly developing field, mainly identifying and quantifying the concentration changes of all the metabolites in a model system or biofluid (such as blood, urine, saliva)[12-15]. It contributes to understanding of disease mechanisms and clinical diagnosis. So we

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investigated and compared serum amino acids metabolism profile of the control mice and the NAFLD mice by using HPLC combined with pattern recognition technology from overall perspective of metabonomics.

In this study, mice fed with high fat and high fructose diet showed some symptoms of NAFLD, such as increase of body weight, liver index and BMI and the rise of serum biochemical level (FBG, TC, CHOL, LDL-C, HDL-C, AST, ALT). Liver histological and hepatic TG and CHOL concentrations further indicated the NAFLD mice model was successful established.

In order to confirm that NAFLD was accociated with amino acids metabolism, we analyzed amino acids metabolic profile of the control group and NAFLD group mice by constructing the PCA and PLS-DA model based on HPLC data. PCA and PLS-DA scores plot showed that samples of these two groups were obviously separated in the two-dimensional spaces. The results also indicated significant differences in serum amino metabolism spectrum. According to VIP value > 1 and a p value < *0.05* of t-test[16], we screened out amino acids with significant differences (glutamic acid, glycine, methionine, isoleucine and valine). Glutamic acid, glycine and methionine participated in glutathione (GSH) metabolism, which is the main defense against oxidant stress and plays an important role in nutrient metabolism[17]. The lack of glutathione contributes to oxidative stress so as to accelerate the development of NAFLD[18]. Our results also showed that the levels of glutamic acid, glycine and methionine were significantly decreased in NAFLD mice compared with the control mice. Isoleucine and valine takes part in BCAAs metabolism, which is acted as nitrogen source in clinical nutrition. Some studies reported BCAAs were not only related with impaired glucose tolerance and obesity but also with blood pressure and dyslipidemia[19]. In our study, the results were consistent with some studies, compared with the control mice, the levels of isoleucine and valine were significantly reduced in the NAFLD mice. In conclusion, all results indicated that these five amino acids may be biological markers of NAFLD. Metabolic profiling analysis can predict metabolic pathways associated with the disease and provide the important data for in-depth study of NAFLD etiology and pathogens.

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