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Study on urine metabolic profiling and pathogenesis of hyperlipidemia

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ABSTRACT

Background: As a recognized risk factor for cardiovascular disease (CVD), hyperlipidemia (HLP) has developed into a high incidence disease that seriously threatens human health. Finding a new target for effective treatment of HLP will be a powerful way to reduce the incidence of CVD. The purpose of this study was to find potential biomarkers in urine of HLP patients and analyze their metabolic pathways to study the pathogenesis of HLP.
Methods: An UPLC-Q-TOF/MS technology was used to detect the metabolites in urine of 60 HLP patients and 60 normal controls. Based on PLS-DA pattern recognition, potential biomarkers related to HLP were screened out.
Results: 22 potential biomarkers related to HLP were identified, which involved amino acid metabolism, fatty acid metabolism, nucleotide metabolism, steroid hormone metabolism and intestinal flora metabolism, and their possible pathogenesis was found to be related to inflammatory reaction and oxidative stress.
Conclusion: The non-targeted metabolomic method based on UPLC-Q-TOF/MS technology can effectively identify potential biomarkers in the urine of HLP patients and explore the possible pathogenesis. Our research will lay a foundation for finding new targets for the treatment of HLP and provide a basis for clinical research on the treatment of HLP.

1. Introduction

Hyperlipidemia (HLP), also known as lipid metabolism disorder or abnormality, refers to a systemic lipid metabolism disorder caused by various reasons, such as high total cholesterol (TC), high triglyceride (TG) and/or low density lipoprotein cholesterol (LDL-C) and/or low high density lipoprotein cholesterol (HDL-C). HLP is a sufficient evidence and a strong risk factor for cardiovascular disease (CVD) and atherosclerosis (AS), and has developed into a high incidence disease that seriously threatens human health [1]. CVD is the leading cause of death for adults in the United States. Compared with the TC level of normal people, people with HLP have about twice the risk of CVD [2]. According to conservative estimates, the increase of TC in the population will lead to an increase of about 9.2 million CVD events in China from 2010 to 2030 [3]. On the one hand, unhealthy eating habits and excessive energy intake make HLP the most prominent and global chronic disease today. On the other hand, HLP is also an important factor leading to AS, fatty liver, cardiovascular and cerebrovascular diseases and other diseases [4]. Therefore, preventing and treating HLP is an effective and common method to reduce the incidence of CVD and other chronic diseases.

Metabolomics is a powerful tool that provides the possibility of studying the differences of small molecule metabolites between different groups at any given time, thus allowing the capture of dynamic physiological conditions corresponding to disease outcomes or metabolic changes [5]. Metabolomic analysis based on ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-Q-TOF/MS) technology can be applied to disease models to identify metabolites with higher resolution and sensitivity effectively and quickly [6].

In recent years, although some progress has been made in the study of HLP with metabolomic techniques, most of them are based on animal models or use clinical samples to analyze certain metabolites. Li et al. [7] found that elevated oxidative stress and energy metabolism disorder during oral glucose tolerance test are important characteristics of metabolic disorder in HLP patients by analyzing serum amino acid and biogenic amine profiles of HLP patients. Wang et al. [8] discovered 9 major metabolites related to lipid metabolism and obesity through the analysis of HLP serum metabolomics. The advantage of using urine samples for non-targeted metabolomic analysis is that, on the one hand, the identification of biochemical components in human urine metabolism group is of high variable importance, and urine has the advantages of non-invasive collection and large sample size [9]. On the other hand,

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non-targeted metabolomic analysis using UPLC-Q-TOF/MS can effectively and quickly identify metabolites with higher resolution and sensitivity and can comprehensively and systematically study the metabolic pathways involved to find new therapeutic targets.

The purpose of this study is to find out the changes of small molecule metabolites in urine of HLP patients and to find potential biomarkers and related metabolic pathways by means of non-targeted metabolomics and metabolomic analysis of urine samples of HLP patients and normal controls. We explored the possible pathogenesis of HLP by metabolomics, laying the foundation for further finding new targets for the treatment of HLP and providing the basis for clinical research on the treatment of HLP.

2. Materials and methods

2.1. Clinical trial registration and ethics statement

This trial was registered in the China Clinical Trial Registration Center with registration number ChiCTR1800016071 and registered in Tianjin University of Traditional Chinese Medicine. The relevant experimental design can be found on this platform. This study has passed the examination by the Ethics Committee of Tianjin University of Traditional Chinese Medicine, with the approval number TJUTCM-EC20180003. Written informed consent was obtained from every recruited patient. This experimental study follows the Helsinki Declaration.

2.2. Subjects

The subjects were all from Tianjin General Hospital and the age, clinical manifestation, TC, TG, LDL-C and HDL-C of each subject were counted. This study consisted of 60 HLP patients and 60 normal controls.

2.3. Standard of the study subject

2.3.1. Diagnostic criteria for the study subject

The diagnostic criteria for subjects were based on the “Guidelines for the Prevention and Treatment of Dyslipidemia in Chinese Adults (Revised Edition 2016)”: plasma TC $\geq$ 6.2 mmol/l (240 mg/dl), TG $\geq$ 2.3 mmol/l (200 mg/dl) or LDL-C $\geq$ 4.1 mmol/l (160 mg/dl) and HDL-C $\leq$ 1.0 mmol/l (40 mg/dl) for adults in fasting 12h [10].

2.3.2. Inclusion and exclusion criteria for the study subject

The inclusion criteria of the subjects were as follows: between 25 and 65 years old, in line with the western medical diagnostic criteria for HLP; although taking lipid-lowering drugs, the drug has been discontinued for more than two weeks, and the blood lipid level still meets the diagnostic criteria.

The exclusion criteria of the subjects were: patients with severe chronic diseases such as coronary artery disease (CAD), diabetes, malignant tumor, kidney disease, rheumatic immune disease, hyperuricemia, hypertension, hypothyroidism, hyperthyroidism, depression, etc; AIDS, syphilis; patients with liver and kidney dysfunction; pregnant or lactating women.

2.4. Clinical sample collection

The subjects were collected fasting morning urine, and centrifuged at 4 °C, 1043 $\times g$ for 15 min to remove the supernatant within 2 h, and then the supernatant was removed and centrifuged at 766 $\times g$ for 8 min at 4 °C. The sample after centrifugation was added with sodium azide in a volume ratio of 100:1 for preservation at −80 °C. All samples were used for the metabolomic analysis.

2.5. Metabolomics analysis

2.5.1. Instruments and reagents

In this study, urine samples (10 μl) were injected into an Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm, Waters) using UPLC-Q-TOF/MS system (Waters Co., Milford, USA) for detection. The column temperature was set at 45°C and the flow rate was 0.3 ml/min. The required reagents were acetonitrile (HPLC Pure, Ocean Pak, Sweden), formic acid (HPLC Pure, Roe, USA) and pure water (Hangzhou Wahaha Group Co., Ltd). The gradient system consisted of 0.1% formic acid in water in mobile phase A and 0.1% formic acid in acetonitrile in mobile phase B (0-8.5 min, A, 99-75%; 8.5-11 min, A, 75-50%; 11-13 min, A, 50-99%; 13-15 min, A, 99%).

MS was performed on a Waters Micro mass Q/TOF micro Synapt High Definition Mass Spectrometer. The electrospray ionization source (ESI source) was used to perform mass spectrometry analysis in positive and negative ionization modes. The MS analysis parameters in positive ionization mode were as follows: ionization source temperature of 120°C, a capillary voltage of 3.0 kV, desolvation temperature of 450°C, drying gas flow rate of 0.3 ml/min, desolvation gas flow rate of 800 l/h and cone gas flow rate of 50 l/h. The MS analysis parameters in negative ionization mode were as follows: ionization source temperature of 110 °C, a capillary voltage of 2.0 kV, desolvation temperature of 450 °C, drying gas flow rate of 0.3 ml/min, desolvation gas flow rate of 800 l/h and cone gas flow rate of 50 l/h. In both positive and negative ionization modes, the quadrupole scanning range was m/z 50 − 1000 Da.

2.5.2. Processing and preparation of samples

After each set of samples was completely melted, centrifugation was carried out at 4°C and 9727 $\times g$ for 10 min. To take 150 μl of supernatant, 150 μl of purified water was added to mix at 1:1, and vortexed to mix. The mixture was centrifuged at 4°C, 16438 $\times g$ for 15 min and the supernatant was injected for UPLC-Q-TOF/MS analysis. 100μl of each urine sample was obtained from each group and mixed as quality control (QC) samples. The preparation of QC samples followed the same procedure with that of the urine samples to validate the experimental precision, repeatability and stability.

2.6. Experimental methodology

Before UPLC-Q-TOF/MS analysis of the sample, QC samples were injected for methodological investigation to verify the experiments, including precision, repeatability and stability.

Instrument precision experiment: A QC sample was injected 6 times consecutively. Twenty chromatographic peaks were selected randomly to calculate RSD values of the areas and retention time of these peaks.

Method repeatability experiment: Six QC samples were prepared in parallel and continuous injection analysis was performed. Twenty chromatographic peaks were randomly selected to calculate RSD values of the areas and retention time of these peaks.

Sample stability test: The same QC sample solution was taken and analyzed at 0, 6, 12, 18, and 24 h. Twenty chromatographic peaks were randomly selected to calculate RSD values of the areas and retention time of these peaks.

2.7. Data analysis

In this study, UPLC-Q-TOF/MS technique was used to perform metabolomic analysis on urine samples of subjects in HLP group and normal controls group (NC group). The data was exported by using Markerlynx software (Waters, Version4.1), and the formed data included retention time, m/z value and normalized peak area. The software parameter is as follows: the error of the mass number is 0.01 Da.

The error of the retention time is 0.5 min, and the S/N is 6. The integrated data were imported into Simca-P 12 statistical software (Umetrics, Sweden) for multivariate statistical analysis. On the basis of partial least squares discriminant analysis (PLS-DA) model, compounds with variable importance plot (VIP) were screened out, and the difference markers were further obtained by *t*-test. Subsequently, they were searched and screened in the HMDB database (http://www.hmdb.ca/), and the final biomarkers were determined according to the fracture information of MS/MS analysis and relevant information such as literature.

3. Results

3.1. Demographic information

Statistical analysis of demographic information was expressed by mean ± standard deviation and processed by SPSS 22.0 software. The indexes between the two groups were compared and tested with two independent samples *t*-text. Values of $P < 0.05$ were considered to have statistical differences, and values of $P < 0.01$ were considered to have significant differences. By analyzing the demographic information of 60 HLP patients and 60 normal controls, the results showed that TG, TC and LDL-C levels in HLP group were significantly higher than those in NC group ($P < 0.01$). In addition, we found that the body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) of HLP group was higher than that of the NC group. The results of each index are shown in Table 1.

3.2. Methodological results

Among the chromatographic peaks obtained in the positive ion mode and the negative ion mode, 20 chromatographic peaks were randomly selected, and RSD values of peak area and retention time were calculated. The results show that the RSD of peak area is less than 15.0 % and the RSD of retention time is less than 1.0 % in both positive and negative ion modes, indicating that the instrument precision, method precision and stability are good.

3.3. Screening and identification of potential biomarkers

UPLC-Q-TOF/MS technique was used to analyze the metabolomics of 120 subjects. In positive and negative ion modes, the base peak intensity (BPI) diagram of QC samples in the urine sample of the subject was shown in Fig. 1. In this study, we used multivariate statistical methods to analyze metabolomic data and used PLS-DA to determine the different metabolites. In positive ion mode and negative ion mode, PLS-DA scores of NC group and HLP group were shown in Fig. 2. In the established PLS-DA model, it can be observed that NC group and HLP group show obvious clustering of classification, and the two groups show a good distinction. In the positive model, the R^2X, R^2Y and Q^2 parameters are 0.158, 0.693 and 0.277, respectively. In the negative model, the R^2X, R^2Y and Q^2 parameters are 0.131, 0.705 and 0.188, respectively. R^2Y and Q^2 can be used to evaluate the model. Generally speaking, the larger R^2Y and Q^2 are, the more stable and reliable the model is. On the basis of PLS-DA model, metabolites that contribute significantly to the classification (VIP > 1) were screened out, and then these markers were tested by *t*-test to obtain the difference markers related to HLP with significant changes ($P < 0.05$) and confirmed by database, MS/MS analysis, literature and other information.

The process of identifying thepotential biomarkers is presented using one substance (RT = 3.68 min, m/z 229.1189) as an example. In the database of HMDB, we searched for the molecular formula of m/z 229.1189, which may be $C_{10}H_{16}N_2O_4$. In addition, in the mass spectrum, the secondary fragments of this substance are m/z 183.1 and 211.1, corresponding to the ion $[M+H]^+$ losing –HCOOH and –H2O. Finally, the compound was identified as prolylhydroxyproline according to the information we obtained from the mass spectrum and database. Other biomarkers are also analyzed according to their fracture rules. Finally, a total of 22 potential biomarkers related to HLP in positive and negative ion modes were screened out, as shown in Table 2.

Table 1
Demographic information

Parameters	NC (n =60)	HLP (n = 60)	P value
Age (years)	40.62 ± 10.69	41.30 ± 9.82	NS
TC (mmol/l)	4.67 ± 0.48	6.42 ± 0.96	0.000
TG (mmol/l)	1.05 ± 0.31	2.21 ± 1.35	0.000
HDL-C (mmol/l)	1.36 ± 0.25	1.30 ± 0.31	0.195
LDL-C (mmol/l)	2.82 ± 0.46	4.23 ± 1.03	0.000
BMI (kg/m^2)	23.33 ± 2.80	24.89 ± 3.10	NS
SBP (mm Hg)	118.34 ± 9.84	122.24 ± 8.09	NS
DBP (mm Hg)	75.71 ± 6.98	78.40 ± 6.48	NS

HLP group compared with NC group, $P < 0.05$ was statistically significant. Abbreviation: NC, normal control; HLP, hyperlipidemia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, not significant.

3.4. Correlation analysis of potential biomarkers

Through the above screening and analysis, 22 potential biomarkers related to HLP were found. In order to further study the correlation between these markers, we conducted a correlation analysis, which helps to explore the pathogenesis of HLP. As shown in Fig. 3, different colors can reflect the degree of correlation between different biomarkers. L-proline, L-isoleucine, glutaric acid, phenylacetic acid and homovanillic acid sulfate have great correlation, and the overall analysis of their correlation plays an important role in amino acid metabolism. In addition, N-phenylacetylphenylalanine and L-glutamine, N-acetyltryptophan and 5-oxoproline, N-acetylglutamic acid and prolylhydroxyproline are closely related to each other, which helps to analyze their role in amino acid metabolism.

3.5. Metabolic pathway analysis of potential biomarkers

In order to further discover the relationship between potential biomarkers of HLP in urine samples and related metabolic pathways involved, we performed MetPA (https://www.metaboanalyst.ca) analysis. As shown in Fig. 4, 22 biomarkers were known to be involved in these metabolic pathways associated with HLP. On one hand, there were many metabolic pathways with an impact value of about 0, indicating that their contribution to the metabolic pathway of HLP is small and will not be considered. On the other hand, the influence value of metabolic pathways is large, indicating that they may be closely related to the pathogenesis of HLP. Through analysis, we found that the main metabolic pathways involved in the body are alanine, aspartate and glutamate metabolism, arginine and proline metabolism, lysine degradation and phenylalanine metabolism. The individual values of these four metabolic pathways were shown in Table 3. The biomarkers involved in these metabolic pathways are L-glutamine, L-proline, N-acetylglutamic acid, glutaric acid and phenylacetic acid. Therefore, we believe that metabolic abnormalities in these four pathways may be closely related to the pathogenesis of HLP.

4. Discussion

In this study, UPLC-Q-TOF/MS was used to investigate the changes of small molecule metabolites in urine of HLP patients and to find potential biomarkers. We screened a total of 22 potential biomarkers related to HLP in positive and negative ion modes. According to the biological analysis of related metabolites such as KEGG data website (https://www.kegg.jp/), we further found that these biomarkers are

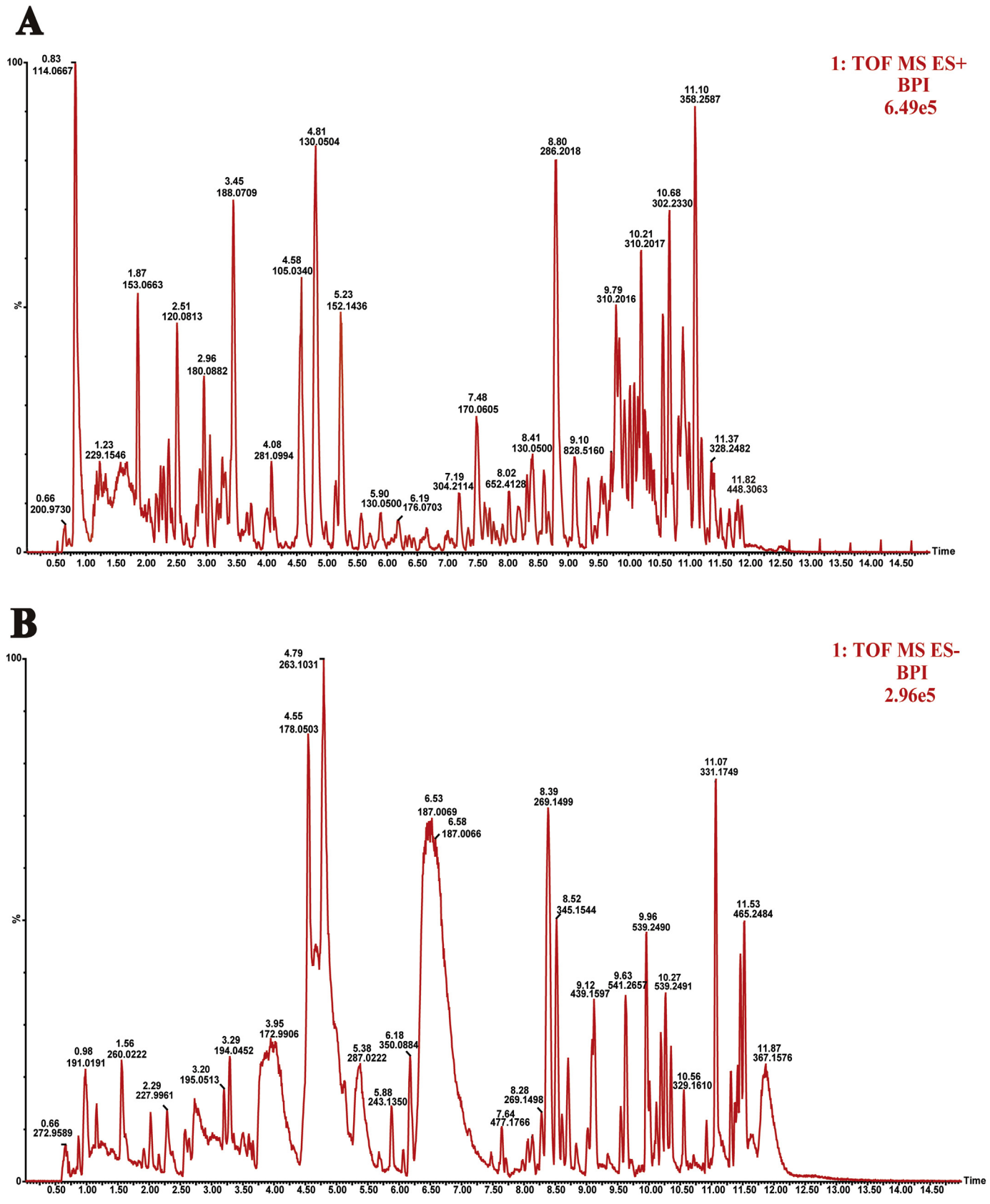

Fig. 1. The base peak intensity (BPI) chromatogram of urine in the QC sample in positive and negative ionization modes gained based on UPLC-Q-TOF/MS.

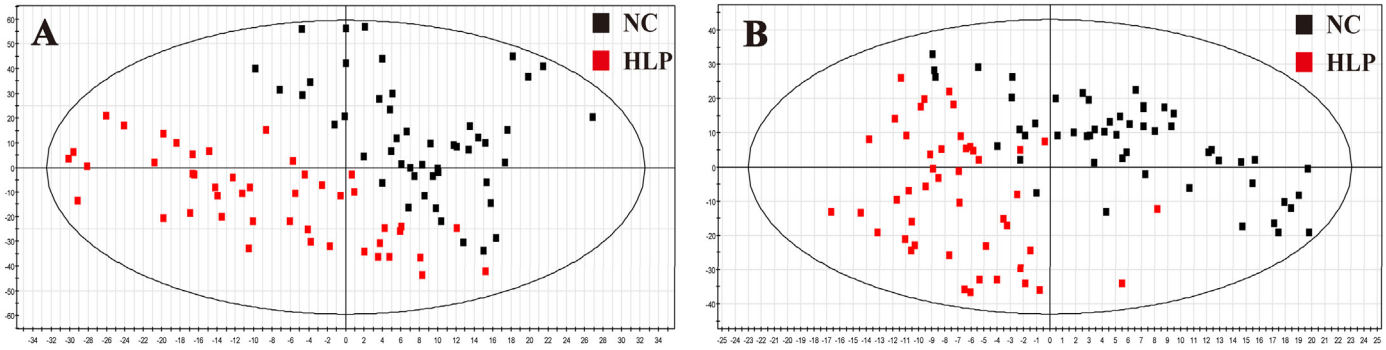

Fig. 2. Results of multivariate statistical analysis. (A) PLS-DA score plot of NC and HLP group in positive ionization mode, (B) PLS-DA score plot of NC and HLP group in negative ionization mode.
Abbreviations: NC, normal controls; HLP, hyperlipidemia.

related to amino acid metabolism, energy metabolism, oxidative stress and nucleotide metabolism, inflammatory reaction and steroid hormone metabolism, and intestinal flora metabolism as shown in Fig. 5.

4.1. Amino acid metabolism

As an important basic substance in the body's life movement, amino acids participate in various energy and substance metabolism. The harm of high-fat diet to liver structure and function has been fully recognized. Experimental and clinical studies have shown that increased fructose intake can lead to liver steatosis, insulin resistance, elevated plasma TG and oxidative stress in the liver, and can change intestinal permeability [11–12]. The protective effect of oral glutamine supplementation on the development of non-alcoholic steatohepatitis mice induced by western diet (rich in fat, fructose and cholesterol) is related to preventing inducible nitric oxide synthase induction and liver lipid peroxidation [13]. Glutamine helps protect intestinal mucosa in the event of inflammation, and in addition to its immunomodulatory properties, it also retains the intestinal barrier by stabilizing the tight junction protein [14–15]. In this study, L-glutamine, 5-oxoproline and L-proline changed in the body of patients with HLP, suggesting that HLP may cause inflammation and oxidative stress, and stimulate intestinal mucosa to change its permeability and cause intestinal barrier dysfunction. In addition, the increased N-acetyl amino acids indicate that the destruction of acetylation activity may affect cell homeostasis through histone-chromatin function and gene regulation, thus causing metabolic imbalance in the body [16–17]. Compared with NC group, the increased levels of N-acetylglutamic acid and N-acetyltryptophan in HLP group indicate acetylation activity in HLP patients may be destroyed and lead to metabolic imbalance.

HLP is a recognized risk factor for CVD and AS. L-arginine can reduce the risk of AS. Lysine can enhance arginine catabolism by activating renal arginase, which in turn consumes arginine levels. Lysine is therefore an antagonist of arginine for health [18]. Glutaric acid found in urine samples of HLP patients in this study can be naturally produced during the metabolism of certain amino acids such as lysine, and the increase in glutaric acid levels suggests that lysine metabolism may be abnormally affected by arginine metabolism, thus increasing the risk of HLP. Inflammation-induced aromatic amino acid metabolism, including phenylalanine metabolism, plays an important role in the regulation of immune function [19]. In this study, it was found that the increase in phenylacetic acid level, which is a metabolite of phenylalanine, may be induced by inflammation caused by HLP, which leads to abnormal phenylalanine metabolism and activation of immune system.

Abnormal amino acid metabolism will inevitably affect protein synthesis, especially branched chain amino acids (BCAAs), which play an important regulatory role in protein, carbohydrate and lipid metabolism [20]. Isoleucine, the BCAAs plays an important role in protein metabolism, including improving sugar metabolism and regulating leptin secretion during food intake [21–22]. Studies have found that BCAAs is positively correlated with blood lipid parameters (TG, TC, LDL-C) [23]. Other experiments have proved that BCAAs supplements can reduce weight gain induced by high fat diet, reduce fat content, activate mammalian target of rapamycin, inhibit liver lipase and reduce liver triglyceride content, however BCAAs reduces weight at the expense of abnormal lipolysis and HLP, resulting in liver lipotoxicity [24]. It can be inferred that abnormal changes in the level of L-isoleucine in the HLP group may cause HLP in this study.

4.2. Energy metabolism

Fatty acids are an important component of all cell membranes, including endothelial cells and cardiomyocytes. Fatty acids and their conjugates are mainly involved in metabolic pathways such as oxidation of saturated fatty acids and peroxidation of unsaturated fatty acids. The disorder of fatty acid metabolism will have a direct impact on the energy metabolism of the body. Clinical and animal studies have shown that reducing dietary saturated fatty acid (SFA) consumption is associated with reducing CVD risk [25]. Some studies have shown that individual SFA may affect CAD risk differently, while most foods rich in SFA contain other components related to CAD risk [26]. Sebacic acid is a saturated, straight-chain naturally occurring dicarboxylic acid. The abnormal changes of sebacic acid level as SFA in this study may affect the level of blood lipid, suggesting that fatty acid oxidation disorders and metabolic disorders in vivo.

4.3. Oxidative stress and nucleotide metabolism

Oxidative stress leads to dysfunction of vascular endothelium, destruction of blood vessel structure and acceleration of AS. The oxidation of LDL is considered to be the main factor in the pathophysiology of AS, and HLP is closely related to AS. Elevated homocysteine level is considered to be a risk factor for AS, which may cause endothelial dysfunction through oxidation of LDL [27–28]. Therefore, we believe that the increase in L-homocystine level in patients with HLP in the form of homocysteine double-bonded may be due to LDL oxidation, which may cause HLP and increase AS risk. Betaine has the function of regulating the expression of apoptosis factor, improving superoxide dismutase activity, and reducing malondialdehyde content, thus increasing the antioxidant activity in cells [29]. In this study, compared with the NC group, the change multiple of betaine in HLP group is 1.39, which indicates that the increase of betaine level in HLP group. This may be because the body is under oxidative stress, and betaine has the function of resisting oxygen free radicals and lipid peroxidation to some extent.

Nucleotide substances can participate in the regulation of vasoconstriction and relaxation through the action of their receptors. Deoxyguanosine, as a nucleoside component of DNA, can be converted into 8-hydroxy-deoxyguanosine (8-OHDG), which is now recognized as

Table 2
Identified metabolites related to hyperlipidemia based on UPLC-Q-TOF/MS

No.	RT (min)	Obsd m/z	Error (ppm)	Parention	Metabolite	MC	Cotent varied	MS/MS	VIP value	*P* value
1	3.68	229.1189	0.44	M + H	Prolylhydroxyproline	1.64	↑**	229.1 183.1 211.1	2.46	0.0045
2	4.92	116.0711	−0.86	M + H	L-Proline	0.66	↓*	116.1 70.1	2.07	0.0498
3	3.51	285.0656	4.91	M + K	N-acetyltryptophan	1.99	↑*	201.1 231.1 183.1	1.95	0.0256
4	3.67	282.1130	0	M-H	N-phenylacetylphenylalanine	0.43	↓**	282.1 264.1	2.73	0.0013
5	5.20	132.1024	−0.76	M + H	L-Isoleucine	1.44	↑*	132.1 86.1 70.1 116.1	1.91	0.0348
6	2.34	269.0617	−4.83	M + H	L-Homocystine	1.61	↑*	223.1 205.1 177.1 148.0	1.75	0.0471
7	2.28	130.0506	−1.54	M + H	5-Oxoproline	1.50	↑*	130.1 84.1	1.74	0.0464
8	5.14	145.0612	−0.69	M-H	L-Glutamine	0.68	↓*	145.1 127.1 99.1	2.07	0.0233
9	1.41	190.0708	−3.68	M + H	N-acetylglutamic acid	1.40	↑*	144.1 154.1 130.1	1.95	0.0359
10	10.39	464.3004	−1.72	M-H	Glycocholic acid	0.71	↓*	464.3 428.3 418.3	1.75	0.0494
11	5.92	150.0553	−1.33	M-H	2-Phenylglycine	0.59	↓*	150.1 132.0 104.1	1.96	0.0236
12	2.70	116.0709	−2.58	M-H	Betaine	1.39	↑*	116.1 59.0	1.93	0.0438
13	5.56	210.0765	−0.48	M + H	Hydroxyphenylacetylglycine	2.12	↑*	210.1 164.1 192.1 135.0	1.80	0.0420
14	5.04	137.0609	4.38	M + H	Phenylacetic acid	1.52	↑*	137.1 91.1 119.1	2.15	0.0132
15	5.06	115.0758	−0.87	M-H	Caproic acid	0.74	↓*	115.1 69.1 99.0 97.1	2.07	0.0194
16	5.55	201.1123	−1.99	M-H	Sebacic acid	0.71	↓*	201.1 183.1	1.77	0.0413
17	2.02	133.0501	0	M + H	Glutaric acid	1.46	↑*	133.1 87.0 115.0 97.0	1.79	0.0450
18	6.34	285.0047	0.70	M + Na	Homovanillic acid sulfate	1.59	↑*	263.0 245.0 181.1 163.0	2.10	0.0252
19	2.59	326.0848	−8.59	M + H	Dihyroxy-1H-indole glucuronide I	2.18	↑**	148.0 132.0	2.78	0.0013
20	2.82	225.0872	−1.33	M-H	Porphobilinogen	1.69	↑*	225.1 207.1 179.1 133.1	1.83	0.0461
21	9.95	347.2218	−1.15	M + H	Cortexolone	1.38	↑*	347.2 311.2 297.2	2.24	0.0102
22	3.77	290.0879	4.83	M + Na	Deoxyguanosine	1.99	↑*	268.1 250.1 232.1	1.95	0.0257

MC, Multiple of change (Relative change level between HLP group and NC group); ↑*, content increased significantly (HLP group compared with NC group, $P < 0.05$); ↑ **, content more significantly increased (HLP group compared with NC group, $P < 0.01$); ↓*, content decreased significantly (HLP group compared with NC group, $P < 0.05$). Abbreviation: NC, normal controls; HLP, hyperlipidemia; RT, retention time.

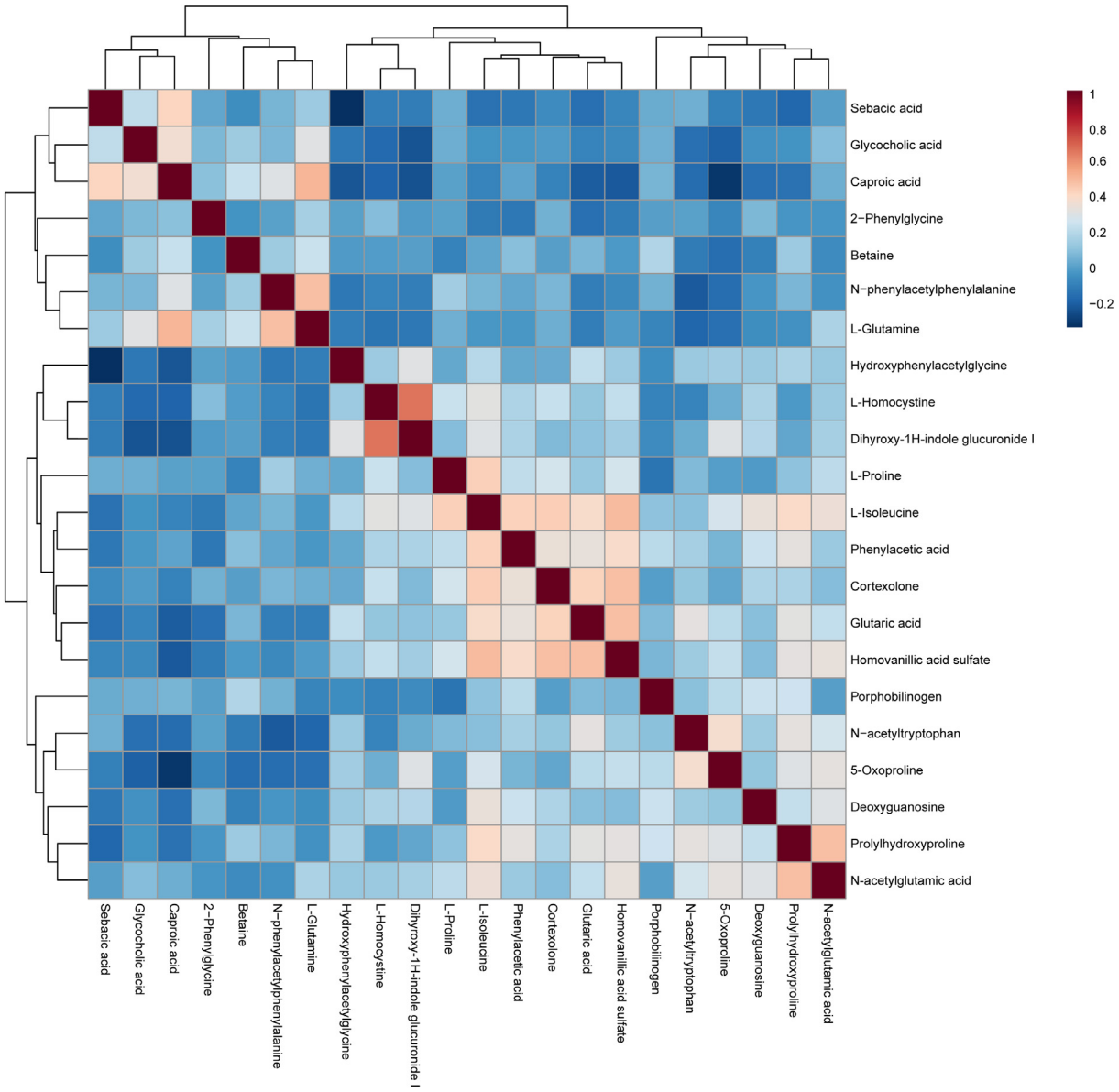

Fig. 3. Correlation analysis of potential biomarkers in human urine with hyperlipidemia.

a new biomarker for evaluating DNA oxidative damage and oxidative stress [30]. In vitro experiments show that folic acid protects ox-LDL induced endothelial dysfunction by reducing reactive oxygen species induced oxidative damage, 8-OHDG content and apoptosis rate. This effect is indirectly caused by the increase of DNA methyltransferase activity, DNA methylation change at the promoter of vascular peroxidase 1 (VPO 1) and the change of VPO 1 expression abundance [31]. In this study, compared with the NC group, the deoxyguanosine in HLP group changes by 1.99 times, and the elevated deoxyguanosine may further cause the elevation of 8-OHDG, and suggest that the body of HLP patients is under oxidative stress and may suffer oxidative damage.

4.4. Inflammation and steroid hormone metabolism

HLP is an important pathogenic factor that induces and promotes AS, and its pathogenesis is complicated. However, the link of inflammatory reaction involved in its pathogenesis has been confirmed definitely. Inflammatory reaction plays a vital role in the stability and progress of AS plaques. Cortexolone is a 17-hydroxycorticosteroid with glucocorticoid (GC) and anti-inflammatory activity. Studies have shown that endogenous GC is an important anti-inflammatory system in vivo. GC signals through GC receptor (GR), which is a ligand-dependent transcription factor of nuclear receptor superfamily, and it is collected to genomic GC response element to regulate gene expression after hormone binding [32]. GR can bind DNA directly and usually activate target genes, including anti-inflammatory mediators [33–34]. GR aslo can bind to other DNA binding regulators, most notably nuclear factor kappa B (NF-κB), thereby inhibiting their pro-inflammatory target genes [35]. The abnormal metabolism of blood fat can cause inflammatory reaction, and the increase of cortexolone content in HLP patients indicates that GCs are activated in the anti-inflammatory system in vivo, thus inhibiting various inflammatory factors to achieve the function of regulating blood fat.

4.5. Intestinal flora metabolism

Intestinal flora plays an important role in the nutrition and metabolism of the host and in protecting the host from external invasion. The occurrence and development of many diseases are also closely related to changes in the structure of intestinal flora. Glycocholic acid is a

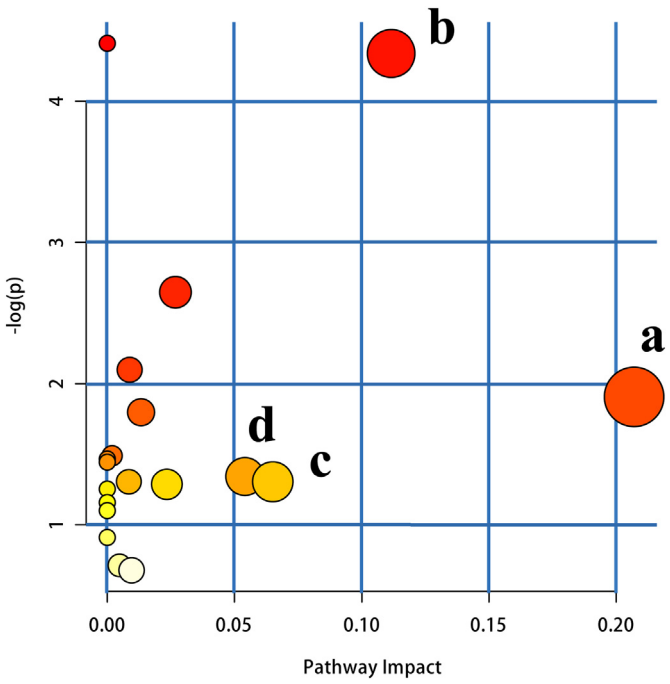

Fig. 4. A summary of pathway analysis with MetPA for hyperlipidemia. a, Alanine, aspartate and glutamate metabolism; b, Arginine and proline metabolism; c, Lysine degradation; d, Phenylalanine metabolism.

Table 3
Result from pathway analysis with MetPA.

Pathway	*P*	−log(*P*)	Impact
Alanine, aspartate and glutamate metabolism	0.14857	1.9067	0.20703
Arginine and proline metabolism	0.01306	4.3385	0.11154
Lysine degradation	0.27131	1.3045	0.06505
Phenylalanine metabolism	0.26134	1.3419	0.05412

The raw *P* is the original *P* value calculated from the enrichment analysis; the impact is the pathway impact value calculated from pathway topology analysis.

conjugate of acylglycine and bile acid glycine. It is a secondary bile acid produced by the action of enzymes present in the microbial flora of the colon environment. At present, studies have found that the mechanism of intestinal flora affecting the change of blood lipid level is mainly as follows: On the one hand, intestinal facultative anaerobic bacteria and anaerobic bacteria affect the subsequent metabolic process by changing the composition and particle size of bile acid metabolism, resulting in the change of blood lipid level [36]. On the other hand, anaerobic bacteria in the cecum and proximal colon ferment non-digestible carbohydrates into short-chain fatty acids, which can be absorbed directly by the intestinal tract [37]. In this study, metabolomic methods were used to find the decrease of glycocholic acid levels in patients with HLP, suggesting that the bile acid metabolism was abnormal, which may be due to the abnormal metabolism of intestinal flora in patients with HLP changing the composition and particles of bile acid metabolism, affecting its normal metabolism and further leading to changes in blood lipid level. The role of intestinal flora imbalance in CVD has been paid more and more attention. It may be a new breakthrough to treat HLP by regulating intestinal flora.

5. Conclusion

HLP has become a prominent and global chronic disease, and the treatment of HLP is an effective way to reduce the incidence of CVD and other chronic diseases, so it is particularly urgent to reveal the pathogenesis of HLP and find new therapeutic targets. In this study, urine samples from HLP patients and normal controls were studied by UPLC-Q-TOF/MS non-targeted metabolomic technique. Through multivariate statistical analysis, 22 potential biomarkers related to HLP were found, which played an important role in amino acid metabolism, energy metabolism, nucleotide metabolism, steroid hormone metabolism and intestinal flora metabolism, and the possible pathogenesis of HLP was found to be related to inflammatory reaction and oxidative stress. Our research lays a foundation for further finding new targets for the treatment of HLP and provides a basis for clinical research on the treatment of HLP.

Conflict of interest

All authors declare that they have no competing interests for this study.

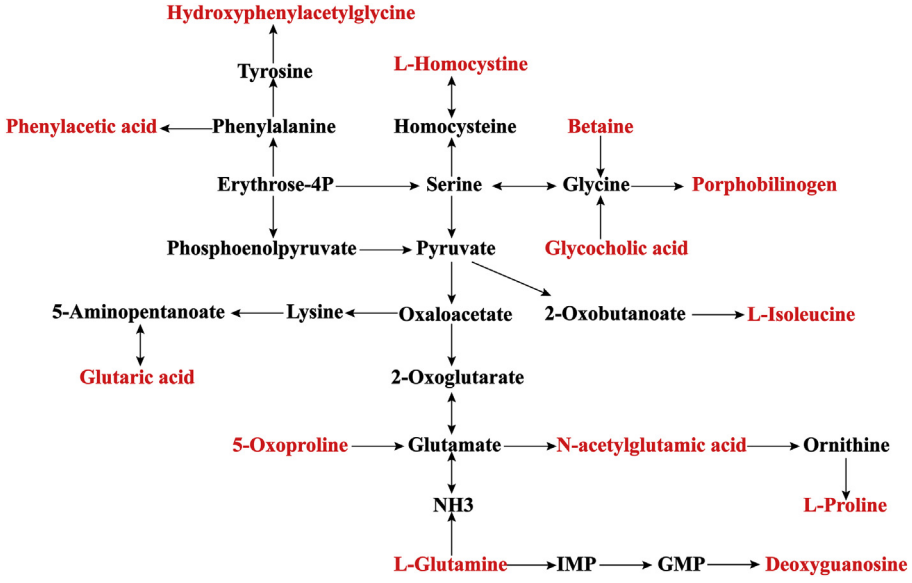

Fig. 5. Metabolic pathway of potential biomarkers in human urine with hyperlipidemia.

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