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Identification of coronary heart disease biomarkers with different severities of coronary stenosis in human urine using non-targeted metabolomics based on UPLC-Q-TOF/MS

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ABSTRACT

Background: Coronary heart disease (CHD) is the leading cause of death worldwide, and its pathogenesis has attracted much attention. Metabolomics serves as an important tool for diagnosing diseases and exploring their pathogenesis in recent years. In this study, CHD patients were studied by comparing them with normal subjects to elucidate biomarkers that are linearly correlated with the severity of coronary stenosis.

Methods: An ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) was used to analyze the urine metabolites of CHD patients and normal subjects. A total of 131 subjects included 27 patients who presented with 50–69% coronary stenosis, 22 with 70–89% stenosis, 29 with 90–99% stenosis, 24 with 100% stenosis, and 29 normal subjects.

Results: A total of 14 potential biomarkers associated with CHD were identified, and among them 4 biomarkers were linearly correlated with the severity of coronary stenosis in CHD patients. The metabolic pathways involved were amino acid metabolism, fatty acid metabolism, energy metabolism, and other pathways.

Conclusion: This study identified the biomarkers and metabolic pathways that may be involved in the occurrence and development of CHD, laying a theoretical foundation for better diagnosis and treatment of CHD in the future.

1. Introduction

Cardiovascular disease is the leading cause of death worldwide. According to the 2019 American Heart Association report [1], the number of deaths due to cardiovascular disease in 2016 was about 17.6 million and is expected to increase to > 23.6 million by 2030, with a mortality rate that exceeds all cancers and chronic lung diseases. Coronary heart disease (CHD) is the most important risk factor of cardiovascular disease, the most important cause of cardiac death, and has become the main health problem for many people [2,3]. CHD refers to heart disease caused by coronary artery atherosclerotic lesions and vascular stenosis or occlusion, resulting in myocardial ischemia, hypoxia, or necrosis [4,5]. The severity of coronary stenosis determines the ischemic symptoms and prognosis. The myocardial blood supply is generally unaffected when vascular stenosis < 50%, and as the degree of vascular stenosis increases, the lack of blood supply leads to chronic stable angina. When atheromatous plaque ruptures, erodes, or bleeds, forming thrombus that blocks the blood vessels, it causes acute myocardial infarction. Therefore, studying CHD together with the severity of coronary stenosis can help predict the severity of CHD through the degree of coronary lesions, which is conducive to an improved diagnosis of CHD as well as aiding in the development of prevention and treatment measures for CHD.

Metabolomics, a newly developed discipline, is an important part of system biology. By quantitative analysis and data processing of small molecule metabolites *in vivo*, the relative relationship between metabolites and physiological and pathological changes is studied to further provide direct data for the exploration of the pathogenesis and pathogenesis of metabolic diseases [6,7]. In the past decade, the progress of technology, the improvement in instruments and data analysis software, and the development of metabolic database have promoted the application of metabolomics in different research fields such as basic science and biomedical and clinical science [8].

In recent years, metabolomics has been increasingly used in the

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study of CHD. For example, Fan et al. studied disturbed metabolic pathways to assess the diagnostic value of metabolomics-based biomarkers in different types of CAD. They used liquid chromatography-quadrupole time-of-flight mass spectrometry to determine the plasma samples of 2324 patients. A total of 89 differential metabolites were identified and 5 metabolic pathways were altered. They believe differences in small-molecule metabolites may reflect underlying CAD and serve as biomarkers for CAD progression [9]. Oni-Orisan et al. used LC/MS/MS to analyze the plasma samples of 162 patients with different severities of coronary stenosis to explore the relationship between circulating epoxyeicosatrienoic acid (EET) levels and CAD extent, and found the presence of obstructive CAD was associated with lower EET metabolite levels [10]. Hasokawa et al. used GC/MS to analyze the serum metabolomic profiles of patients hospitalized for follow-up coronary angiography 6 months after stent implantation; 23 patients were restenotic ≥75%, 47 were restenotic ≤50%, and 16 were de novo atherosclerotic. Isobutylamine and 8 other biomarkers were eventually discovered in the major restenosis group which significantly different from those in the minor restenosis group. These differences might imply possible changes in the activated metabolic pathways [11]. Li et al. used UHPLC-Q-TOF/MS to analyze the blood samples from 150 patients with at least 1 major coronary artery with ≥80% stenosis and 150 normal controls with ≤20% stenosis and discovered palmitic acid, linoleic acid, and other 6 biomarkers had the strongest correlation with CHD, which may be the biomarkers that are potentially involved in the pathogenesis of CHD [12]. Most of the studies found in the literature used blood as a biological sample in CHD metabolomic research. However, using urine is hugely advantageous, as it is the final product of human metabolism and therefore the best matrix for a systematic analysis [13], with the advantages of easy collection, non-invasive procedure, and large sample sizes. For example, Zhou et al. analyzed the urine of 1072 patients with different syndrome types of CHD using UPLC-Q-TOF/MS and found 15 CHD phlegm and blood stasis (PBS) syndrome biomarkers and 12 CHD *qi* and *yin* deficiency (QYD) syndrome biomarkers [14]. Wang et al. used UPLC-Q-TOF/MS to analyze the urine samples of acute coronary syndrome (ACS) and healthy controls and found that 9 markers were upregulated and 11 markers were downregulated. They also showed that fatty acid metabolism, fatty acid beta-oxidation metabolism, amino acid metabolism, and the TCA cycle played important roles in ACS [15]. Therefore, it is necessary to study the potential biomarkers in the urine of patients with CHD to increase our knowledge of CHD.

In this study, we applied non-targeted metabolomics using UPLC-Q-TOF/MS. A case-control study design was used to compare the urine samples of CHD patients with different severities of coronary stenosis, with healthy subjects used as the control. The aim of this study was to investigate the changes in small molecule metabolites in urine in CHD patients with varying severity of coronary stenosis in order to find potential biomarkers and related metabolic pathways and explore whether these biomarkers have a linear trend in patients with different severities of coronary stenosis to provide a theoretical basis for the clinical prevention and control of CHD.

2. Materials and methods

2.1. Study subjects

In this study, 131 study subjects were recruited from five clinical medical units. According to the coronary angiography or coronary CTA examination results, CHD patients were divided into four groups according to at least one coronary artery (including the left main artery, left anterior descending artery, left circumflex artery, and right coronary artery) and their main branch arterial stenosis. A total of 27 patients had 50–69% stenosis (G1 group), 22 patients had 70–89% stenosis (G2 group), 29 patients had 90–99% stenosis (G3 group), 24 patients had 100% stenosis (G4 group), and 29 were normal subjects (NS group).

This experimental study follows the Helsinki Declaration and was registered with the China Clinical Trial Registration Center, registration number ChiCTR-ROC-17013957, and the registered unit is Tianjin University of Traditional Chinese Medicine. All of the subjects were given informed consent prior to inclusion in the study.

2.2. Reagents and materials

This study used the following reagents and materials: acetonitrile (Sigma-Aldrich); formic acid (ROE); pure water (Hangzhou Wahaha Group Co., Ltd); Waters Acquity UPLC (Waters Co.); and an Acquity UPLC BEH C18 chromatographic column (2.1 mm × 100 mm, 1.7 μm, Waters).

2.3. The standard for the study subjects

2.3.1. Diagnostic criteria for the study subjects

The diagnostic criteria of the CHD patients were from the American College of Cardiology (ACC) and American Heart Association (AHA) Revised Version of the CHD Diagnosis Guidelines and the 2007 Guidelines for the Diagnosis and Treatment of Chronic Stable Angina in China. The diagnostic criteria are as follows: (1) a clear history of old myocardial infarction, (2) previous coronary angiography or coronary CTA examination showing that at least one of the major branches of coronary artery stenosis had a diameter ≥50%, and (3) had undergone coronary revascularization (PCI or CABG) treatment.

2.3.2. Inclusion and exclusion criteria for the study subjects

The inclusion criteria for the study subjects were between the ages of 30–80 years old and meeting the diagnostic criteria for CHD. The exclusion criteria were as follows: history of myocardial infarction or unstable angina within 3 months or underwent coronary revascularization (surgical bypass surgery or angioplasty); combined with another heart disease, psychoneurosis, menopausal syndrome, hyperthyroidism, spinal cord, or vertebral artery syndrome cervical spondylosis; gastroesophageal reflux disease or hiatal hernia and another disease that may cause chest pain; the use of β-receptor blockers, calcium channel antagonists, energy metabolism drugs, nitrates, or more than three of the four classes of drugs; patients with hypertension who still had high blood pressure after antihypertensive drug treatment (systolic blood pressure ≥160 mmHg and diastolic blood pressure ≥100 mmHg), severe cardiopulmonary insufficiency, and severe arrhythmia (rapid atrial fibrillation, atrial flutter, and paroxysmal ventricular tachycardia, etc.); active liver disease, or with unexplained elevated serum transaminase, or ALT, AST > 2 times the upper limit of the normal reference value; renal dysfunction; severe primary diseases such as hematopoietic system or malignant tumor; pregnant, lactating women or women of childbearing age with fertility requirements; those with mental illness or cognitive impairment; those with severe metabolic diseases such as diabetic nephropathy and gouty nephropathy; and those who participated in other clinical trials in the prior 3 months. The researchers concluded that there were other conditions that were not appropriate for this study.

2.4. Clinical sample collection

The subjects' fasting morning urine was collected and rapidly centrifuged at 1043 × *g* for 15 min at 4 °C. The supernatant was removed and centrifuged at 766 × *g* for 8 min at 4 °C, after which the supernatant was added with sodium azide at a volume ratio of 100:1 for corrosion prevention. All of the samples were refrigerated at −80 °C.

Table 1
Demographic information.

Parameters	NS	G1	G2	G3	G4
n	29	27	22	29	24
Sex (male/female)	15/14	11/16	10/12	19/10	22/2
Age (male), y	51.7 ± 6.6	64.8 ± 8.9	64.1 ± 6.6	61.4 ± 7.8	65.3 ± 7.9
Age (female), y	47.9 ± 2.5	65.6 ± 6.8	67.8 ± 4.8	67.6 ± 5.6	66.5 ± 6.4
Height, cm	165.9 ± 7.6	164.8 ± 6.5	166.5 ± 8.2	168.1 ± 7.9	170.7 ± 6.1
Weight, kg	65.5 ± 10.5	68.3 ± 8.8	71.9 ± 13.5	72.3 ± 9.4	74.8 ± 11.9
SBP, mmHg	121.7 ± 9.3	133.5 ± 12.0	134.6 ± 16.3	133.3 ± 21.4	141.4 ± 19.2
DBP, mmHg	76.5 ± 5.3	76.7 ± 12.3	76.3 ± 9.0	77.3 ± 12.5	82.9 ± 13.9

Data presented as average value ± SD. NS, normal subjects; G1, 50–69% stenosis; G2, 70–89% stenosis; G3, 90–99% stenosis; G4, 100% stenosis; SBP, systolic blood pressure; DBP, diastolic blood pressure.

2.5. Metabolomics analysis

2.5.1. Processing and preparation of samples

After each group of samples completely thawed, 300 μL of each was centrifuged at 4 °C, 9727 ×*g* for 10 min. Then 150 μL of the supernatant was mixed with the same volume of distilled water and vortexed for 1 min using a vortex machine. The supernatant was centrifuged at 16,438 ×*g* for 15 min, and 200 μL of the supernatant was placed in a sample vial for UPLC-Q-TOF/MS analysis.

In addition, 100 μL of each urine sample from each group was pipetted into a centrifuge tube to prepare a QC sample for UPLC-Q-TOF/MS methodology study, which was prepared in the same manner as each sample.

2.5.2. Instruments and reagents

Data acquisition was performed on a UPLC-Q-TOF/MS system (Waters). UPLC analysis was performed in a Water Acquity UPLC system. Urine samples (10 μL) were injected into an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters). The column temperature was set at 45 °C, and the flow rate was 0.3 ml/min. 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%; and 13–15 min, A, 99%).

MS was performed on a Waters Micro Mass Q/TOF Micro Synapt high-definition mass spectrometer. An electrospray ionization source (ESI source) was used for mass spectrometric detection in the positive and negative ionization mode. The MS analysis parameters were as follows: a capillary voltage of 3.0 kV, drying gas temperature of 325 °C, drying gas flow of 0.26 mL/min, desolvation gas flow of 700 L/h, source temperature of 120 °C, desolvation temperature of 450 °C, and cone gas flow of 50 L/h. The reference ion ($[M + H]^+$ = 556.2771, $[M + H]^-$ = 554.2615) was used to ensure accuracy in the spectral acquisition, and the quadrupole scan range was *m/z* 50–1000 Da.

2.6. Experimental methodology

2.6.1. Instrument precision test

Using the same QC sample solution continuously injected 6 times, 20 chromatographic peaks were randomly selected to calculate the RSD values of the peak area and retention time.

2.6.2. Repeatability test

Six QC samples were prepared in parallel, continuous injection analysis was performed, and 20 chromatographic peaks were randomly selected to calculate the RSD values of the peak area and retention time.

2.6.3. Sample stability test

The same QC sample solution was analyzed at 0, 6, 12, 18, and 24 h, and 20 chromatographic peaks were randomly selected to calculate the RSD values of the peak area and retention time.

2.7. Data analysis

In this study, UPLC-Q-TOF/MS (Waters) technology was used to perform metabolomic analysis of urine samples from normal subjects and CHD subjects. The specific methods of data processing and metabolomics analysis were as follows: the original data collected from the workstation were exported using MarkerLynx software (Waters, version 4.1). Then the data were imported into SIMICA-P + 12.0 software (Umetrics AB) after 80% revision to establish the principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) models. On the basis of the PLS-DA model, metabolites with variable importance plots (VIP > 1) were screened as potential biomarkers using the *t*-test to screen out substances with $P < 0.05$ as potential differential metabolites of CHD. Subsequently, the *m/z* values of the candidate biomarkers were searched in the HMDB database (http://www.hmdb.ca/), the value of each parameter was set as the ion mode: positive/negative; adduct type: M + H/M + K/M + Na/M-H; and molecular weight tolerance: ± 0.01 Da. The final biomarkers were further determined based on the MS/MS analysis, metabolite database information, and literature information.

3. Results

3.1. Demographic information

The statistical analysis of the demographic information was performed using SPSS (version 21.0) and was expressed as the average value ± SD. The indexes of the 2 groups of data were compared and 2 independent sample *t*-tests were applied (Table 1).

3.2. Metabolomic analysis

3.2.1. Experimental methodology

The results of the experimental methodology are shown in Table S1. The instrument precision, method repeatability, and sample stability were all in line with the requirements of metabolomics.

3.2.2. Data processing

The UPLC-Q-TOF/MS analysis technique was used to perform the metabolomic analysis on 131 subjects. In the positive and negative ionization mode, the base peak intensity (BPI) chromatogram of the urine in the QC sample is shown in Fig. S1.

In this study, the data on the urine samples collected via UPLC-Q-TOF/MS were processed using the multivariate pattern recognition method for metabolomics research. First, SIMCA-P + 12.0 multivariate statistical analysis software (Waters, version 4.1) was used to establish an unsupervised PCA model for the positive and negative ion mode data of the NS, G1, G2, G3, and G4 groups. See the supplementary material Fig. S2. Then a supervised metabolomics analysis was further carried out using PLS-DA. The PLS-DA score map in Fig. 1 demonstrates that under the two ionic modes, all of the groups showed good aggregation

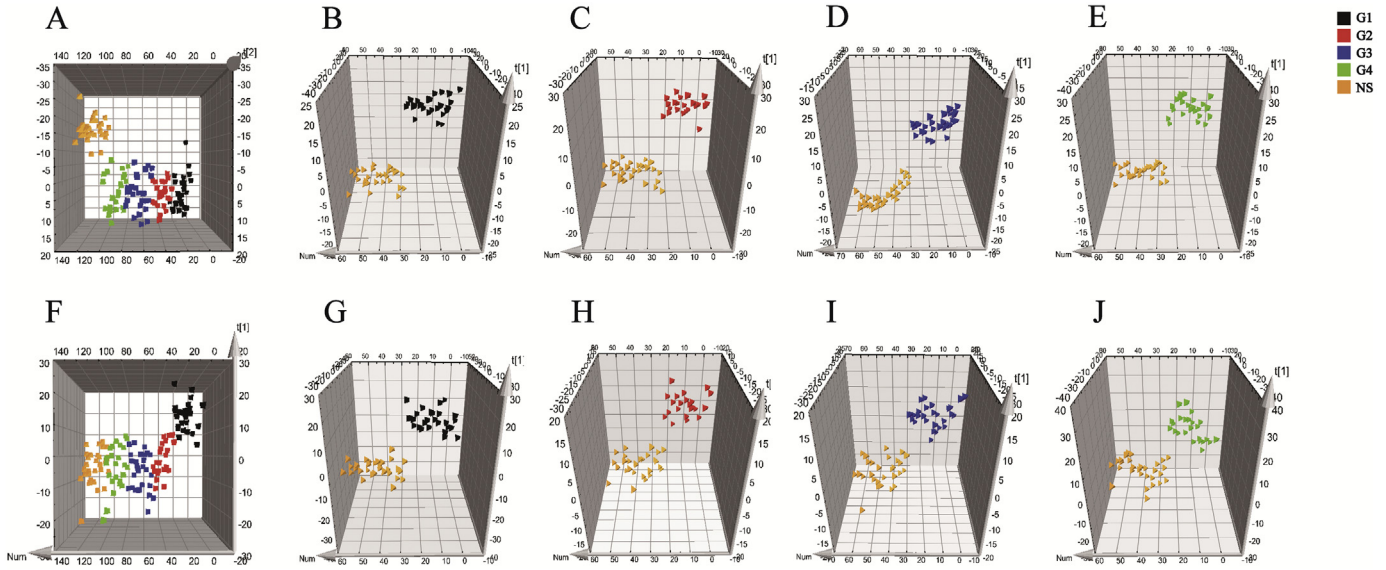

Fig. 1. PLS-DA score plots of the multivariate statistical analysis.
Positive: (A) PLS-DA score plots of the G1, G2, G3, and G4 groups compared with the NS group. (B) PLS-DA score plots of the G1 group compared with the NS group. (C) PLS-DA score plots of the G2 group compared with the NS group. (D) PLS-DA score plots of the G3 group compared with the NS group. (E) PLS-DA score plots of the G4 group compared with the NS group. Negative: (F) PLS-DA score plots of the G1, G2, G3, and G4 groups compared with the NS group. (G) PLS-DA score plots of the G1 group compared with the NS group. (H) PLS-DA score plots of the G2 group compared with the NS group. (I) PLS-DA score plots of the G3 group compared with the NS group. (J) PLS-DA score plots of the G4 group compared with the NS group.

and classification, and there was a significant separation between the CHD groups and the NS group. These indicate that the endogenous substances in the patients changed compared with the NS group, and the specific manifestation was the difference in the metabolites.

3.2.3. Identification of the potential biomarkers

Based on the PLS-DA model, substances with significant differences ($P < 0.05$) between the CHD group and the NS group were screened using the *t*-test. Then the *m/z* values of these substances were searched in the HMDB database (http://www.hmdb.ca/) for retrieval comparison and comparison screening and to identify potential differential metabolic molecules in CHD. The adjustment error was 0.01 Da. We identified a total of 14 substances by comparing the mass spectrometry secondary fragments of the compounds in the HMDB database. The 14 metabolic small molecules were used as biomarkers for CHD (Table 2).

3.2.4. ROC curve analysis of the potential biomarkers

Using the aforementioned experiments, we found potential biomarkers for the diagnosis of CHD. We used a ROC curve to verify whether these biomarkers had diagnostic significance. ROC curves are widely used to evaluate the sensitivity and specificity of biomarkers, and the relationship between the sensitivity and specificity can be observed intuitively [16]. The area under the curve (AUC) corresponding to each curve can judge the diagnostic efficiency of biomarkers, and the larger the area, the higher the value of the judgment [17]. The data of all metabolites in the CHD group and NS group were analyzed via binary logistic regression using SPSS software (version 21.0) to obtain the predictive probability. Then the AUC value was obtained using ROC curve analysis to judge the overall diagnostic efficacy of these 14 markers for CHD. Fig. 2 shows that the AUC of the biomarkers in the CHD patients was as high as 0.977, indicating that the biomarkers we selected had extremely high diagnostic significance.

3.2.5. Analysis of the metabolic pathways of the potential biomarkers

In order to further reveal the relationship between the small molecule metabolites of CHD, the HMDB ID of the 14 identified biomarkers of CHD were introduced into MetPA (https://www.metaboanalyst.ca/) for a metabolic pathway analysis. The value of their contribution to the metabolic pathways of CHD is that these metabolic pathways are considered to be related to the pathogenesis of CHD. The MetPA analysis results of this experiment are shown in Fig. 3. The pathways involved in this experiment are arginine and proline metabolism; valine, leucine, and isoleucine biosynthesis; tryptophan metabolism; and purine metabolism. Thus, the amino acid metabolism pathway is the main pathway of metabolic disorder in CHD and may be the main cause of CHD.

3.2.6. Correlation analysis of the potential biomarkers

We also assessed the relationship between 14 biomarkers with significant differences associated with CHD (Fig. 4). Different colors can reflect the magnitude of the correlation between various metabolites. From the overall observation, the 14 biomarkers were more closely related, which is of good significance to explain the pathogenesis of CHD. Among them, leucyl-phenylalanine, adenosine, and 5-hydroxyindoleacetaldehyde had a substantial correlation; Isobutyrylcarnitine and isoleucylproline were highly correlated. At the same time, the correlation between *N*-acetyl-b-D-galactosamine, adipic acid, and 4-hydroxyproline, kynurenine, prolyhydroxyproline, and 2-isopropylmalic acid were also good. These relationships indicate that amino acid metabolism and energy metabolism can work together in CHD through a certain mechanism, which has good guiding significance for our in-depth analysis of the pathogenesis of CHD.

3.3. Linear correlation analysis of the potential biomarkers

Using the five groups of subjects as the abscissa and the levels of the biomarkers as the ordinates, a line chart was created (Fig. 5). The line chart indicates that the trends in biomarker variations have three classes: class (I)has a linear relationship with the severity of coronary stenosis. The relative level of these substances increases or decreases with the increase in the severity of coronary stenosis. Class (II)has no significant change in CHD with the severity of coronary stenosis and basically maintains the same level. Class (III)has no relationship with the severity of coronary stenosis. This type of substance exhibits volatility and does not show a significant correlation with the severity of coronary stenosis.

According to the aforementioned rules, a class (I)substance has 2-

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

No.	$t_{R(min)}$	Obsd m/z	Compound	Molecular formula	Parent ion	Content varied	Fold change	MS/MS
1	3.2	231.0747	Kynurenine	$C_{10}H_{12}N_2O_3$	M + Na	↓*	0.7	209.0 191.0 174.0 163.0
2	6.2	198.0529	5-Hydroxyindoleacetaldehyde	$C_{10}H_9NO_2$	M + Na	↑*	2.1	176.0 158.0 132.0
3	4.8	177.0766	2-Isopropylmalic acid	$C_7H_{12}O_5$	M + H	↓**	0.5	177.0 131.0 85.0
5	2.4	132.0666	4-Hydroxyproline	$C_5H_9NO_3$	M + H	↓*	0.7	132.0 114.0 86.0
6	5.2	306.0620	Adenosine	$C_{10}H_{13}N_5O_4$	M + K	↑**	2.0	268.1 251.0 250.0 232.0
7	3.1	233.1639	Isobutyrylcarnitine	$C_{11}H_{22}NO_4$	M + H	↑**	1.7	214.1 187.1 60.08
8	11.4	301.1508	Leucyl-phenylalanine	$C_{15}H_{22}N_2O_3$	M + Na	↑*	3.0	279.1 262.1 261.1 233.1
9	3.5	160.0385	Indole-3-carboxylic acid	$C_9H_7NO_2$	M-H	↓*	0.7	160.0 142.0
10	3.3	194.0436	4-hydroxyhippuric acid	$C_9H_9NO_4$	M-H	↑**	2.4	194.0 176.0 148.0
11	10.2	251.1346	Isoleucylproline	$C_{11}H_{20}N_2O_3$	M + Na	↑**	2.5	229.1 211.1 183.1
12	3.5	147.0675	Adipic acid	$C_6H_{10}O_4$	M + H	↓*	0.7	147.0 129.0 101.0 55.0
13	1.6	222.1007	N-Acetyl-b-D-galactosamine	$C_8H_{15}NO_6$	M + H	↓*	0.5	222.0 206.0 190.0
14	2.5	251.1041	Prolylhydroxyproline	$C_{10}H_{16}N_2O_4$	M + Na	↓*	0.6	229.1 211.1 183.1
15	1.1	181.0640	Nicotinuric acid	$C_8H_8N_2O_3$	M + H	↓*	0.6	181.0 163.0 135.0

↑, Content increased significantly; ↓, content decreased significantly; $^*P < 0.05$; $^{**}P < 0.01$ (the CHD group compared with the NS group).
Fold change (the CHD group compared with the NS group).

isopropylmalic acid, 5-hydroxyindoleacetaldehyde, 4-hydroxyhippuric acid, and adenosine; a class (II)substance has nicotinuric acid and prolylhydroxyproline; and a class (III)substance has *N*-acetyl-b-D-galactosamine, adipic acid, isobutyrylcarnitine, and some amino acid metabolism substances.

4. Discussion

In this study, UPLC-Q-TOF/MS technology was used to investigate the changes in the urinary small molecule metabolites in patients with CHD to elucidate potential biomarkers. We screened 14 biomarkers of CHD, and 4 biomarkers such as 4-hydroxyhippuric acid showed a linear trend in patients with different severities of coronary stenosis. According to the biological analysis of related metabolites found by data sites such as KEGG (http://www.genome.jp/kegg/), these small molecule metabolites are mainly concentrated in amino acid metabolism, fatty acid metabolism, energy metabolism, nucleotide metabolism and nicotinic acid metabolism pathways. We believe that metabolic abnormalities in these pathways may be the main cause of CHD.

4.1. Amino acid metabolism

Amino acids and their derivatives, among the most important basic substances in the life of organisms, play an important role in human metabolism. Moreover, amino acid metabolism as a precursor of energy metabolism can increase ATP formation [18]. It has been reported that the abnormality in amino acid metabolism is related to cardiovascular diseases and is one of the basic metabolic pathways in vivo [19–21]. In this study, two biomarkers with linear trends appeared simultaneously in the amino acid metabolic pathway, which also suggests that amino acid metabolism is the focus of CHD.

4-hydroxyhippuric acid is the product of hippuric acid. In this study, its level in the patients with CHD showed a linear trend. Previous studies have reported that when the level of 4-hydroxyhippuric acid *in vivo* increases, the amount of hippuric acid increases and endothelial cell dysfunction occurs, resulting in the improper regulation of vascular tension, activation of the inflammatory response, and accelerating thrombosis, which is also a key step in the development of atherosclerosis [22]. 5-hydroxyindoleacetaldehyde is a product of serotonin metabolism, which is one of the tryptophan metabolic pathways [23]. Serotonin is a hormone that plays a number of roles in the vascular

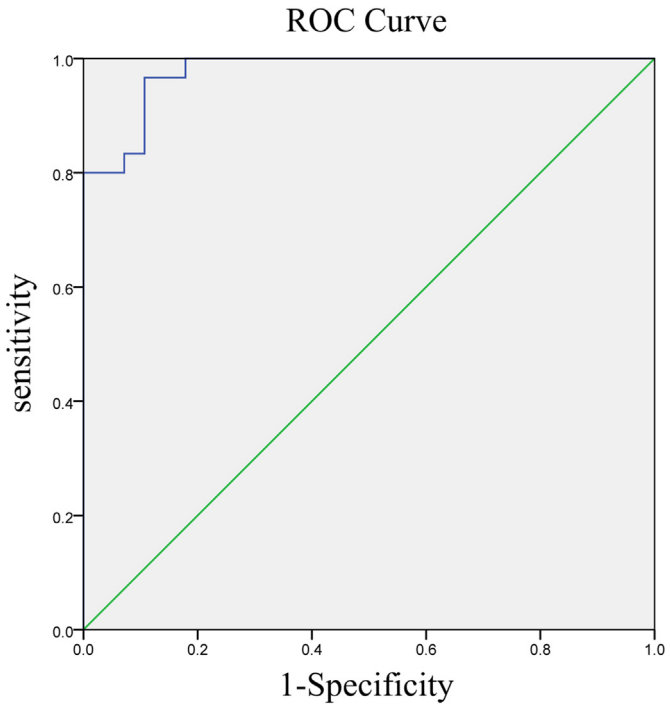

Fig. 2. ROC curve analysis of the biomarkers for CHD.

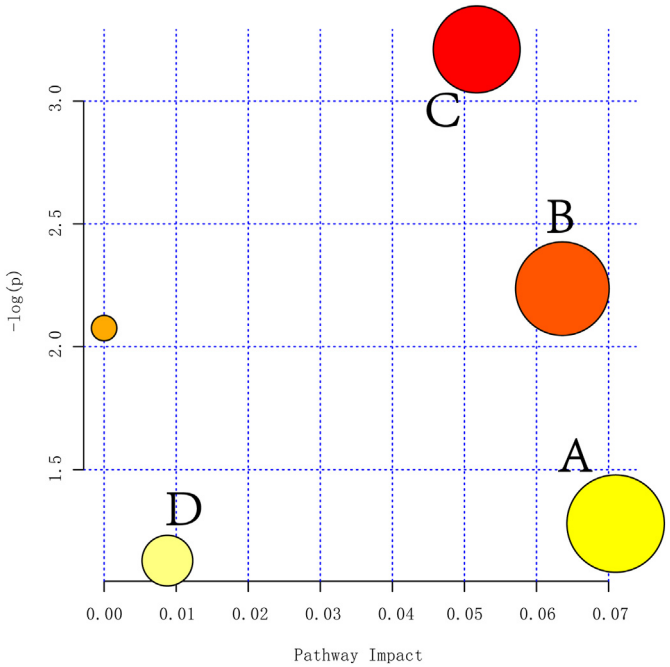

Fig. 3. A summary of pathway analysis with MetPA for CHD. (A) Arginine and proline metabolism. (B) Valine, leucine, and isoleucine biosynthesis. (C) Tryptophan metabolism. (D) Purine metabolism.

system in promoting atherosclerosis, including vasoconstriction, platelet aggregation and thrombosis [24–26]. In this study, the 5-hydroxyindoleacetaldehyde level showed an increasing trend with the increase in coronary stenosis, indicating that the increase in its content will lead to the aggravation of CHD, which is important.

Although the other 6 biomarkers showed no linear trends, their levels were significantly different from those of the normal subjects. The kynurenine pathway is another metabolic pathway of tryptophan. It has recently been found that the enhancement of the tryptophan and kynurenine pathways is related to the occurrence of cardiovascular disease. Kynurenine is produced by tryptophan under the action of indoleamine 2,3-dioxygenase (IDO). Interleukin γ, as the strongest inducer of IDO, mediates inflammation in the body, leading to atherosclerosis [27,28]. It was also confirmed that indole-3-carboxylic acid, a metabolite of indole-tryptophan, is closely related to the occurrence of CHD [29]. Prolylhydroxyproline is a dipeptide formed by the dehydration condensation of proline and hydroxyproline and is a biomarker of collagen degradation [30]. When myocardial fibrosis occurs after myocardial infarction in patients with CHD, abnormal synthesis of myocardial interstitial collagen increases and degradation decreases, which is consistent with the result that the level of prolylhydroxyproline in CHD patients was lower than in the normal subjects in this study. Isoleucylproline is a dipeptide composed of phenylalanine and proline, and proline and 4-hydroxyproline are the main components of collagen [31]. Collagen is an important component of atherosclerotic plaque and can evaluate the degree of fibrosis. The increased abnormal synthesis of interstitial collagen after myocardial infarction in CHD patients leads to myocardial fibrosis, which also suggests that prolylhydroxyproline and 4-hydroxyproline may play an important role in the pathogenesis of CHD. Leucyl-phenylalanine is a dipeptide composed of leucine and phenylalanine. Tyrosine is a metabolite of phenylalanine. The levels of three amino acids (tyrosine, phenylalanine, and isoleucine) strongly predict the risk of future type 2 diabetes in related research [32]. Many epidemiology studies demonstrate that type 2 diabetes is the most important risk factor for CHD [33,34], also indicating that leucyl-phenylalanine plays an important role in the pathogenesis of CHD.

4.2. Fatty acid metabolism

Fatty acids are the main functional substances of the heart, accounting for 40–80% of its energy source [15]. Fatty acids are mainly metabolized by beta oxidation. Fatty acids are first activated by acetyl-CoA synthetase to produce acetyl-CoA and are then transported to the mitochondria for beta oxidation decomposition. In this experiment, the levels of fatty acids such as adipic acid and 2-isopropylmalic acid in the patients with CHD were lower than those in the normal subjects, indicating that the oxidation of fatty acids was enhanced and the level of non-oxidized fatty acids was decreased. In addition, the amount of 2-isopropylmalic acid showed a linear trend in the CHD patients, meaning that its level would affect the occurrence and development of CHD. Studies have shown that because of myocardial ischemia and hypoxia in patients with CHD, the intracellular energy receptor AMP-activated protein kinase (AMPK) is activated at this time, which can regulate the uptake and oxidation of fatty acids [35]. In cardiomyocytes, FAT/CD36 is a key enzyme for fatty acid transport, and studies have shown that the activation of AMPK contributes to the promotion of fatty acid uptake in cardiac myocytes and is closely related to the fatty acid displacement transferase CD36 [36].

4.3. Energy metabolism

CHD metabolomic studies have confirmed that CHD is related to abnormal carnitine metabolism [37,38]. Normal contractile and diastolic activity of the heart requires myocardial cells to provide enormous capacity, which is obtained by transporting fatty acids to mitochondria through carnitine for beta oxidation. When energy metabolism is insufficient, dysfunction of myocardial cell activity and cardiac blood uptake lead to myocardial ischemia and then to CHD. In this study, the expression level of isobutyrylcarnitine was significantly upregulated in the patients with CHD, which also verified that the energy metabolism disorder in CHD caused the accumulation of carnitine.

Glucose is also one of the energy sources of the myocardium. *N*-acetyl-b-D-galactosamine is an oligosaccharide residue that exists in the mucosa, glandular epithelium, and blood vessels. In this study, its level was significantly downregulated in the patients with CHD, indicating

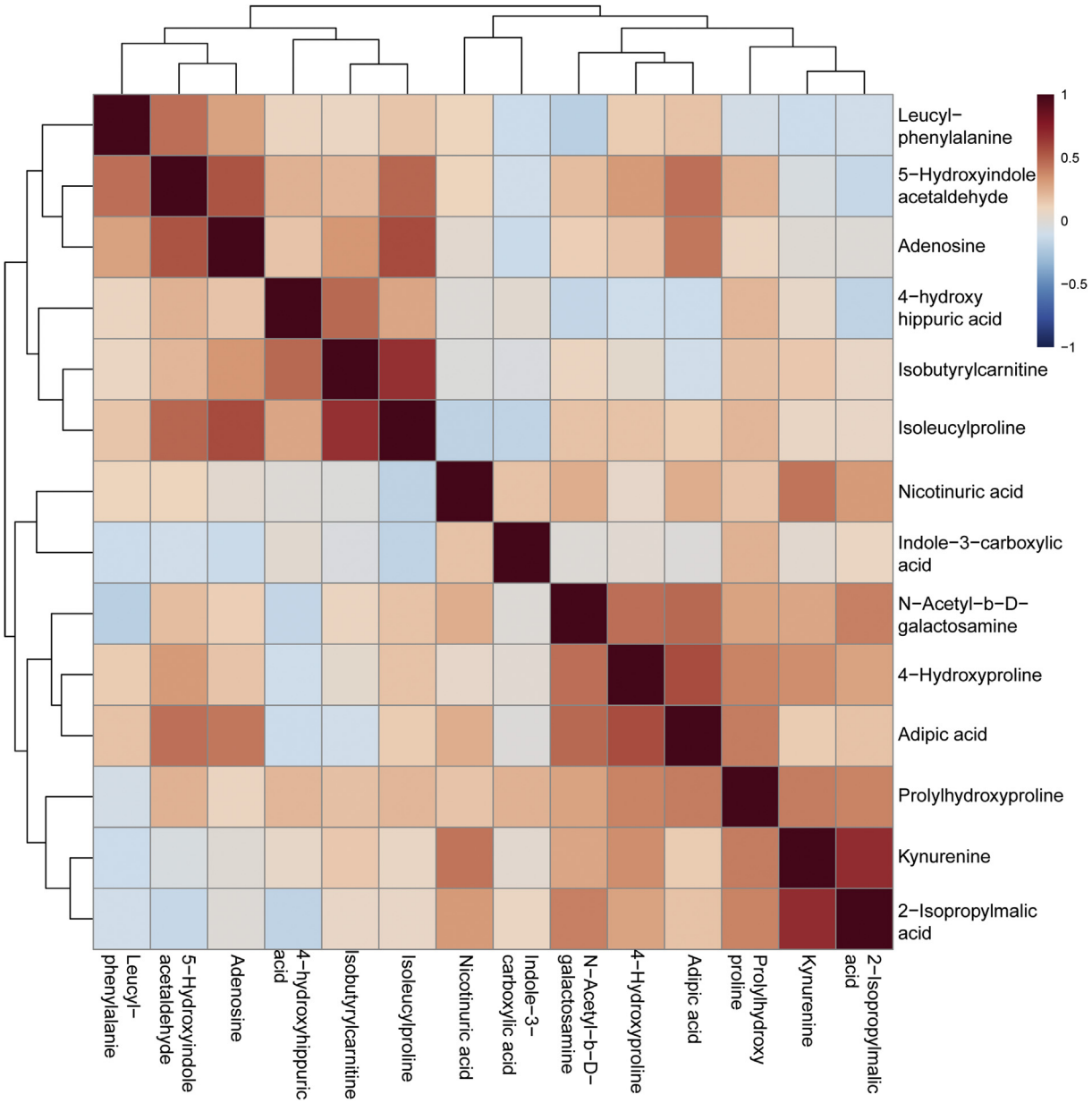

Fig. 4. Correlation analysis of the biomarkers for CHD.

glucose metabolism disorder. Studies have shown that glucose become the main energy source of myocardial energy metabolism during myocardial ischemia [39], and glucose enters cells through glucose transporter (GLUT) proteins on the cell membrane. GLUT4 is the main GLUT of cardiomyocytes [40]. Its intrinsic activity affects glucose uptake, and its increased expression and enhanced myocardial glucose metabolism have protective effects on ischemic myocardium. Other studies have shown that glycosaminoglycan composed of *N*-acetyl-b-D-galactosamine has anticoagulant and antithrombotic effects [41]. The main pathological basis of CHD is that intracoronary thrombosis leads to the vascular stenosis, and the blood flow becomes slow or interrupted. Therefore, the amount of *N*-acetyl-b-D-galactosamine in this study was significantly reduced in the CHD patients.

4.4. Nucleotide metabolism and nicotinic acid metabolism

Adenosine is a purine nucleoside that acts primarily by binding to receptors of the cardiac myocytes and vascular endothelial cells. The experimental data show that the activation of the adenosine A2 receptor has a myocardial protective effect [42,43]. When adenosine binds to the A2 receptor of the vascular endothelium and smooth muscle, it can produce significant vasodilator effects, increase coronary blood flow, and protect the heart. The experiment found that the level of adenosine in the CHD patients was significantly higher than in the normal subjects, but it decreased with the increased stenosis and aggravation of the disease. The mechanism involved is worthy of further discussion.

This study also found abnormal nicotinic acid metabolism in the CHD patients. Nicotinuric acid is a metabolite of nicotinic acid that is formed by the combination of nicotinic acid and glycine. Nicotinic acid is a water-soluble vitamin that can inhibit the release of free fatty acids in adipose tissue, reduce the synthesis rate of low-density lipoprotein and very low density lipoprotein, reduce triglycerides and total cholesterol, and increase high-density lipoprotein. It is commonly used in the treatment of atherosclerosis and can inhibit the formation of atherosclerosis [44,45], and thus decelerating the occurrence and development of CHD.

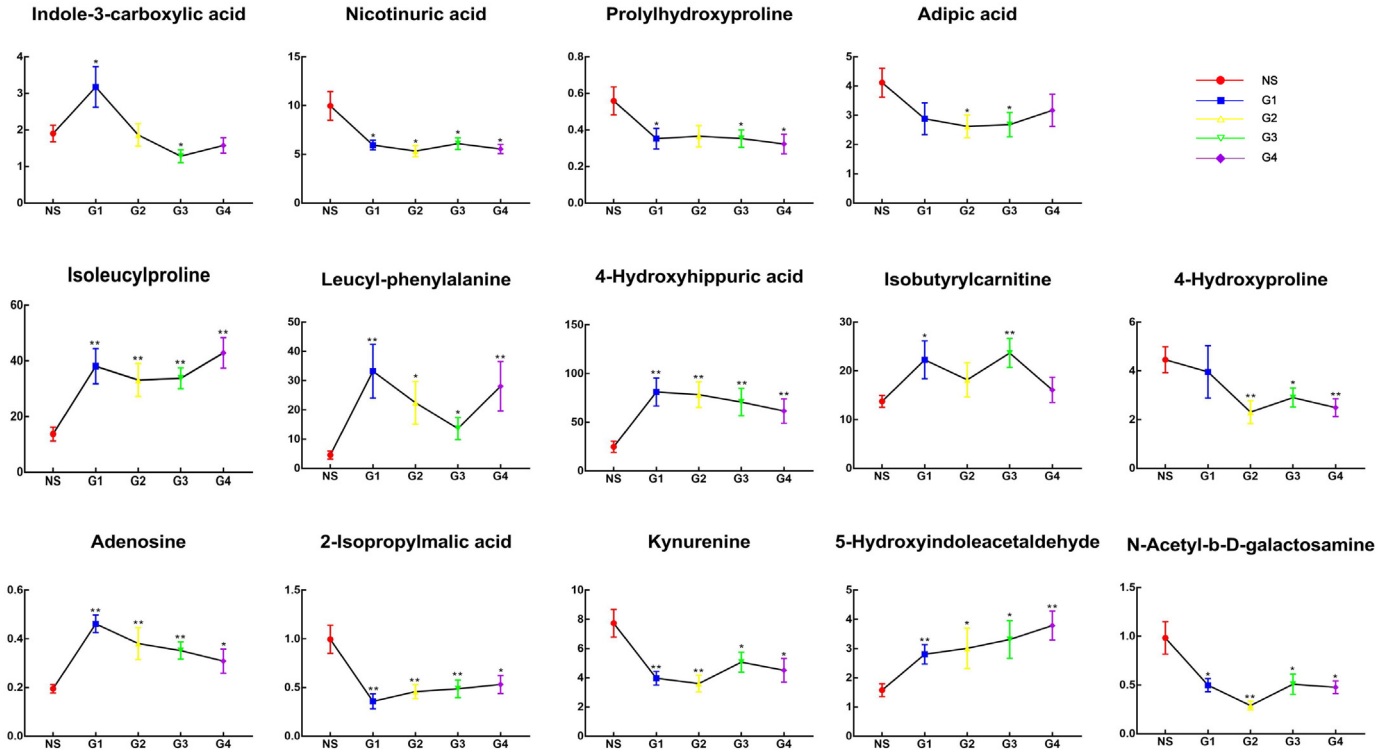

Fig. 5. Line chart of the biomarkers for CHD content change.
X axis, the five groups of subjects; Y axis, the relative content of each marker; NS, normal subjects; G1, 50–69% stenosis; G2, 70–89% stenosis; G3, 90–99% stenosis; G4, 100% stenosis; $^{*}P < 0.05$; $^{**}P < 0.01$ (compared with the NS group).

5. Conclusion

Overall, this study analyzed the metabolites in the urine of CHD patients with different severities of coronary stenosis and normal subjects using non-targeted UPLC-Q-TOF/MS metabolomics. Experiments found 14 biomarkers closely correlated with CHD that are mainly involved in multiple metabolic pathways: amino acid metabolism, fatty acid metabolism, energy metabolism, nucleotide metabolism and nicotinic acid metabolism. Amino acid metabolism, as a precursor of energy metabolism, can increase the formation of ATP and play an important role through the 5-hydroxytryptamine and kynurenine pathways. Fatty acid metabolism is the main way for the myocardium to obtain energy. Nucleotide metabolism also plays an extremely important role in cell energy metabolism, among which adenosine functions by combining the production of myocardial cells and vascular endothelial cell receptors. Thus, these pathway metabolic disorders jointly promote the occurrence and development of CHD. Among them, 2-isopropylmalic acid, 5-hydroxyindoleacetaldehyde, 4-hydroxyhippuric acid, and adenosine showed a good linear relationship in the CHD patients. These substances to some extent mediated the occurrence and development of CHD and were closely related to the degree of development of CHD. To determine the relationship between the levels of these 4 biomarkers and the severity of CHD, we can better understand the role of these biomarkers in the onset and progression of CHD, which is of great importance for assessing the condition of CHD and judging the prognosis. At the same time, metabolomics, as a newly emerging technology of systemic biology, has broad research and application prospects for studying the pathophysiological process of disease occurrence and the development and search for new biomarkers of disease. The difference between this report and other metabolomics studies of CHD is that we analyzed the metabolic differences between patients with different degrees of coronary artery stenosis and normal subjects to identify key linear markers. The levels of these markers are positively or negatively correlated with the degree of coronary artery stenosis. Focusing on the level of these markers in patients with early CHD may contribute to the early clinical prevention and treatment of CHD. This study also provides experimental data for the clinical evaluation of CHD, which is of guiding significance for in-depth understanding of the pathogenesis of CHD.

Disclosure of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.07.017.

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