Untargeted metabolomics analysis of differences in metabolite levels in congenital heart disease of varying severity

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Research Article

Keywords: Congenital heart disease, Severity, Metabolomics, Differential metabolites

Posted Date: February 8th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2464935/v1

Abstract

Background

Congenital heart disease (CHD) is characterized by various phenotypes, however, differences in metabolic profiles associated with CHD of various severity have not been elucidated. In this study, differences in metabolite concentrations among mild, moderate, and severe forms of CHD were explored, providing novel clues for our understanding of the mechanism of CHD.

Methods

Maternal amniotic fluid samples from fetuses with mild (n = 15), moderate (n = 7), and severe (n = 29) CHD lesions were analyzed by GC-TOF/MS. PCA, PLS-DA, and differential metabolite analysis among these three groups were conducted.

Results

PCA and PLS-DA models showed that metabolic profiles were comparable among CHD of different severity. Significant differences between mild and moderate CHD lesions were observed in the levels of gluconolactone, ornithine, threonine, sorbose, pentadecanoic acid, and the uric acid/xanthine ratio. Of these six differential metabolites, gluconolactone (r = 0.469, *P* = 0.028), sorbose (r = 0.577, *P* = 0.005) and the uric acid/xanthine ratio (r = 0.438, *P* = 0.041) were positively correlated with moderate CHD lesions, while ornithine (r=-0.531, *P* = 0.011), threonine (r=-0.546, *P* = 0.009), and pentadecanoic acid (r=-0.454, *P* = 0.034) were negatively associated. We found 9 differential metabolites between mild and severe CHD lesions, among which the alpha-ketoisovaleric acid/valine ratio (r=-0.383, *P* = 0.010), gluconolactone (r = 0.391, *P* = 0.009), and 4-hydroxycinnamic acid (r = 0.342, *P* = 0.023) were correlated with severe CHD lesions. Only sorbose showed significant differences between moderate and severe CHD lesions, and was negatively associated with severe CHD lesions (r=-0.341, *P* = 0.042).

Conclusions

Compared with mild CHD, specific differences were observed in metabolites or metabolite ratios in moderate and severe CHD lesions of CHD, several of which were significantly correlated with CHD severity. These results can help to understand the metabolic status of the affected fetus and provide new possibilities for exploring the pathological mechanism of CHD.

Background

Congenital heart disease (CHD) is the most common birth defect, with a prevalence of approximately 10 per 1,000 live births. CHD is characterized by various phenotypes, for example, ventricular septal defect

(VSD), atrial septal defect (ASD), tetralogy of Fallot(TOF), persistent truncus arteriosus and double outlet right ventricle DORV [1, 2]. CHD may cause miscarriage, premature delivery, low birth weight, or death [3]. In addition, CHD can result in an impairment of brain growth and development, such as a delay in cortical development or white matter injury [4, 5]. The impact of CHD on the fetus varies with its severity. CHD with mild lesions, such as VSD with a spontaneous closure ratio of 40–60%, has relatively limited consequences on patients' health [6–8]. However, severe CHD, such as left heart dysplasia, transposition of the great arteries, truncus arteriosus, heterotaxia, double-outlet right ventricle, can result in death either during or after birth [9]. Currently, the differences in the metabolic status among fetuses in the second trimester of pregnancy with different types of CHD are unknown and are worth further exploration.

The composition of the pool of metabolites is the result of the interaction of biological and environmental factors and holds a large amount of information that is capable of reflecting phenotypes. Metabolomics aims to characterize different types of small molecule metabolites from different sample types, and untargeted metabolomics methods focus on detecting a larger number of possible metabolites [10]. Therefore, metabolomics has become a powerful platform for novel technologies; it is an interdisciplinary discipline that combines biochemistry, analytical chemistry, bioinformatics, and statistics [11]. The unique advantages of metabolomics helped identify disease biomarkers, and promoted progress in toxicological and pharmacokinetic studies [12, 13].

Previous reports were mainly focused on metabolic differences between normal fetuses and those with CHD, but few studies addressed the variation of metabolic profiles with different CHD severity [14–16]. The present investigation utilized untargeted metabolomics methods to explore the metabolic profiles and differential metabolites associated with mild, moderate, and severe CHD lesions. Our findings may advance the understanding of the metabolic status of the fetus affected by CHD with various degrees of lesions and provide novel concepts for studying the pathological mechanism of CHD with different severity.

Methods

Samples

This study was approved by the Medical Ethics Committee of Nanjing Maternity and Child Health Care Hospital. From the 1859 samples collected from January 2012 to December 2018, maternal amniotic fluid from fetuses affected by CHD with mild (15 cases), moderate (7 cases), and severe (29 cases) lesions were selected for untargeted metabolomics analysis. Table 1 shows the specific types of the selected 51 CHD cases. The severity of CHD lesions was classified according to published literature [17–19]. There was no significant difference in gestational age, maternal age, and fetus sex among the three groups with different CHD severity (Table 2). All specimens were residual samples left after clinical molecular diagnostic tests and were stored at -80°C before analysis. All cases were single pregnancies from the Jiangsu province diagnosed with CHD by fetal echocardiogram and then referred to the prenatal diagnosis center of the Nanjing Maternity and Child Health Care Hospital. Except for CHD, other structural

or chromosomal abnormalities and pregnancy complications, such as hypertension and diabetes, were excluded from all samples. Except for 28 terminated pregnancies, postnatal ultrasound diagnosis was consistent with prenatal results in the remaining 23 cases of CHD.

Table 1
Specific CHD types of mild, moderate, and severe lesions groups

Group	CHD type	Number of cases
Mild lesions	VSD	15
Moderate lesions	TOF	5
	PVS + VSD + ASD	1
	PVS + VSD	1
Severe lesions	Heterotaxy	4
	IAA,B	2
	IAA,B + VSD	3
	IAA,B + VSD + ASD	1
	IAA,B + MA	1
	DORV + VSD	3
	DORV + PVS + VSD	1
	d-TGA + VSD	4
	HLHS	1
	HLHS + VSD	1
	HLHS + APVR	1
	HLHS + VSD + MA	1
	HLHS + MA + DORV + mid-aortic dysplasia	1
	Persistent truncus arteriosus + VSD	2
	TOF + PA	1
	TOF + interruption of inferior vena cava	1
	Severe PA + mitral insufficiency	1

VSD, ventricular septal defect; ASD, atrial septal defect; TOF, tetralogy of fallot;

PVS, pulmonary valve stenosis; IAA, B, interrupted aortic arch type B; MA, mitral atresia;

DORV, double outlet right ventricle; d-TGA, d-transposition of the great arteries;

HLHS, hypoplastic left heart syndrome; APVR, partial anomalous pulmonary venous return;

PA, pulmonary atresia.

Table 2
Demographic characteristics of samples used for metabolic profile analysis

Subject characteristics	Mild lesions	Moderate lesions	Severe lesions	*P* value
Number of samples	15	7	29	
Gestational age (weeks)	23(21–25)	25(22–26)	24(20–26)	0.169
Maternal age (years)	29(24–39)	30(22–32)	28(23–37)	0.421
Fetal gender(female/male)	6/9	1/6	12/17	0.399

Gestational age and maternal age are represented by median.

Reagents and instruments

Ultrapure water was prepared using a Milli-Q reference system (Millipore, Billerica, MA, USA). Fatty acid methyl ester (C7–C30, FAMEs) standards, anhydrous sodium sulfate, methoxyamine HCl, and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (Optima LC-MS), N-methyl-N(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% (vol/vol) trimethylchlorosilane (TMCS), hexane, methanol (Optima LC-MS), chloroform, dichloromethane, and acetone were obtained from Thermo-Fisher Scientific (Fair Lawn, NJ, USA). A Rxi-5 ms capillary column (30 m × 250 μm i.d., 0.25 μm film thickness, Restek Corporation, Bellefonte, PA, USA) was used for separation. GC-TOF/MS (Pegasus HT, Leco Corp., St. Joseph, MO, USA), equipped with an Agilent 7890B gas chromatograph (Agilent, Santa Clara, CA, USA) and a Gerstel multipurpose sampler MPS2 with dual heads (Gerstel, Muehlheim, Germany) was applied for sample analysis.

Sample preparation and analysis conditions for GC/TOF-MS

Sample preparation, mass spectrometry conditions, and detection methods of GC/TOF-MS were as we previously reported [14] .

Data analysis

XploreMET 3.0 software was applied for peak picking, automated baseline denoising, signal alignment, and deconvolution. Metabolites were annotated by comparing mass spectrometry data and retention indices with the JiaLib metabolite database. After transforming each data into a comparable data vector, MetaboAnalyst 5.0, an open software, was used for metabolomics analysis, which included the principal component analysis (PCA), partial least square discrimination analysis (PLS-DA), and variable importance in the projection (VIP) value. Mann-Whitney *U* tests, Student's *t*-tests, and Spearman correlation analyses were performed using SPSS 22.0. Metabolites shown by univariate analysis to have

P < 0.05 and VIP > 1 on multivariate analysis were considered reliable differential metabolites [20]. For Spearman correlation analysis, *P* < 0.05 was considered significant [21].

Results

Absence of significant differences in metabolic profiles among CHD with mild, moderate, and severe lesions

A total of 265 small molecule metabolites were detected in amniotic fluid, and 178 metabolites were identified successfully. We performed multivariate statistical analysis of the 178 metabolites and 50 metabolite ratios based on the KEGG database.

An unsupervised PCA model showed no significant separation trend among CHD with mild, moderate, and severe lesions (Fig. 1A). Moreover, using a supervised PLS-DA model, we confirmed the lack of significant differences in metabolic profiles between the three groups (Fig. 1B). In the PLS-DA model, although a separation trend could be observed among the three groups ($R^2Y = 0.90$, $Q^2 = -0.36$), a negative value of Q^2 indicated that the model was over-fitted. In fact, the results of the permutation test of this model yielded the *P*-value of 0.673, demonstrating that the separation trend was caused by the over-fitting of the model. These results showed no significant difference in metabolic profiles among CHD with mild, moderate, and severe lesions.

Differential metabolite levels among CHD with mild, moderate, and severe lesions

Although the overall metabolic profile was comparable between the three groups, univariate and multivariate analyses were conducted to identify the differential metabolites among the three groups. Comparison of differential metabolites between each two groups was performed by Student's *t*-test or the Mann-Whitney *U* test. Normally distributed data were analyzed by *t*-tests while if one or more of the two datasets were non-normally distributed, the Mann-Whitney *U* test was used. VIP values were obtained according to the PLS-DA model. The identified differential metabolites between the different groups are shown in Table 3.

Table 3
Differential metabolites of CHD with different lesions and the trend of change

Group	Metabolites or metabolite ratios	*P* value	*t* value	*Z* value	VIP value	Trend in the latter
mild lesions VS moderate lesions	Ornithine	0.002	2.789		1.37	↓
	Threonine	0.005	0.26		1.68	↓
	Gluconolactone	0.007	-2.162		2.58	↑
	Sorbose	0.008		-2.643	1.76	↑
	Uridine/Cytidine ratio	0.016	-2.621		0.69	↑
	Pentadecanoic acid	0.033	2.289		1.69	↓
	Putrescine/Ornithine ratio	0.041	-2.183		0.99	↑
	Uric acid/Xanthine ratio	0.041	-2.186		1.97	↑
	D-Xylose/D-Xylitol ratio	0.045		-2.009	0.66	↑
mild lesions VS severe lesions	Tryptamine	0.014	-2.556		0.55	↑
	Alpha-ketoisovaleric acid/L-Valine ratio	0.012		-2.513	2.99	↓
	Gluconolactone	0.022	-2.382		2.58	↑
	4-Hydroxycinnamic acid	0.024	-2.338		2.41	↑
	Ornithine	0.026	2.313		1.37	↓
	Linoleic acid	0.035	2.175		2.03	↓
	Xanthine/Xanthosine ratio	0.041	2.113		2.40	↓
	Hippuric acid	0.037	2.149		2.04	↓
	4-Hydroxyproline	0.042	1.769		0.16	↓
	MG182	0.047	2.045		2.2	↓
moderate lesions VS severe lesions	Uridine/Cytidine ratio	0.002	3.368		0.69	↓
	Sorbose	0.049	2.043		1.76	↓

VIP, variable importance in the projectio

The results of the univariate analysis indicated that the concentrations of 9 metabolites or metabolite ratios, namely, ornithine, threonine, gluconolactone, sorbose, the pentadecanoic acid, uridine/cytidine ratio, the putrescine/ornithine ratio, the uric acid/xanthine ratio, and the D-xylose/D-xylitol ratio differed significantly in mild and moderate lesions. Of these, ornithine, threonine, gluconolactone, sorbose,

pentadecanoic acid, and the uric acid/xanthine ratio met the VIP > 1 requirement, indicating that these 6 metabolites or metabolite ratios were more reliable. The levels of ornithine, threonine, and pentadecanoic acid were significantly reduced in the moderate CHD lesions, while the levels of gluconolactone, sorbose, and the uric acid/xanthine ratio levels were significantly elevated.

The univariate analysis showed that the levels of 10 metabolites or metabolite ratios differed significantly between mild and severe CHD. These were ornithine, gluconolactone, tryptamine, linoleic acid, hippuric acid, 4-hydroxycinnamic acid, 4-hydroxyproline, MG182, the alpha-ketoisovaleric acid/valine ratio, and the xanthine/xanthosine ratio, with only tryptamine and 4-hydroxyproline not satisfying the VIP > 1 criterion. Of the 8 differential metabolites verified by multivariate statistical analysis, only the levels of gluconolactone and 4-hydroxycinnamic acid were increased in severe CHD, while those of the other metabolites were decreased.

There was significant difference in sorbose levels and the uridine/cytidine ratio between moderate and severe CHD; however, only sorbose was verified by the multivariate analysis, showing a decrease.

In general, although there were no significant differences in the overall metabolic profile among mild, moderate, and severe CHD, there were still several metabolite levels that differed between groups. However, we did not identify a metabolite that was able to distinguish between mild, moderate, and severe CHD at the same time.

Correlation analysis between differential metabolites and CHD severity

Spearman correlation analysis was performed to analyze the associations between differential metabolites and CHD severity (Table 4). Gluconolactone (r = 0.469, P = 0.028), sorbose (r = 0.577, P = 0.005), and the uric acid/xanthine ratio (r = 0.438, P = 0.041) were positively correlated with moderate CHD lesions, while ornithine (r=-0.531, P = 0.011), threonine (r=-0.546, P = 0.009), and pentadecanoic acid (r=-0.454, P = 0.034) were negatively associated with moderate CHD lesions in comparison with mild lesions. Among the 6 metabolites, ornithine, threonine, and sorbose showed moderate correlations, while gluconolactone, pentadecanoic acid, and the uric acid/xanthine ratio showed lower associations. When mild and severe CHD lesions were compared, we found several differential metabolites that were significantly correlated with severe CHD, including the alpha-ketoisovaleric acid/valine ratio (r=-0.383, P = 0.010), gluconolactone (r = 0.391, P = 0.009), and 4-hydroxycinnamic acid (r = 0.342, P = 0.023); however, the degree of correlation was low. Compared with moderate CHD lesions, we found only one differential metabolite, sorbose (r=-0.341, P = 0.042), to be significantly associated with severe CHD; however, the correlation coefficient was low.

Table 4
Spearman correlation analysis of differential metabolites and the severity of CHD

Group	Metabolites or metabolite ratios	*P* value	Correlation Coefficient
mild lesions VS moderate lesions	Ornithine	0.011	-0.531
	Threonine	0.009	-0.546
	Gluconolactone	0.028	0.469
	Sorbose	0.005	0.577
	Pentadecanoic acid	0.034	-0.454
	Uric acid/Xanthine ratio	0.041	0.438
mild lesions VS severe lesions	Alpha-ketoisovaleric acid/Valine ratio	0.010	-0.383
	Gluconolactone	0.009	0.391
	4-Hydroxycinnamic acid	0.023	0.342
	Ornithine	0.051	-0.296
	Linoleic acid	0.061	-0.285
	Xanthine/Xanthosine ratio	0.061	-0.285
	Hippuric acid	0.061	-0.285
	MG182	0.117	-0.240
moderate lesions VS severe lesions	Sorbose	0.042	-0.341

Overall, ornithine, threonine, sorbose, gluconolactone, pentadecanoic acid, and the uric acid/xanthine ratio were correlated with moderate CHD lesions, while the alpha-ketoisovaleric acid/valine ratio, gluconolactone, 4-hydroxycinnamic acid, and sorbose were correlated with severe CHD lesions.

Discussion

CHD is the most common birth defect, and its phenotype varies widely. However, metabolic profiles in CHD of different severity were rarely investigated. In this study, GC-TOF/MS-based untargeted metabolomics was used to explore the metabolic profile of CHD with mild, moderate, and severe lesions, and the differences in metabolite concentrations among the three groups were determined. Multivariate analysis models, such as PCA and PLS-DA, did not identify any significant differences in metabolic profiles among mild, moderate, and severe types of CHD. However, univariate and multivariate statistical analysis showed that the levels of gluconolactone, threonine, ornithine, sorbose, pentadecanoic acid and the uric acid/xanthine ratio were statistically different between the mild and moderate lesions of CHD,

while the alpha-ketoisovaleric acid/valine ratio, ornithine, gluconolactone, linoleic acid, hippuric acid, 4-hydroxycinnamic acid, MG182, and the xanthine/xanthosine ratio levels were significantly different between mild and severe CHD lesions. Only one differential metabolite, sorbose, was identified between moderate and severe CHD lesions. Of the identified differential metabolites, ornithine, threonine, sorbose, gluconolactone, pentadecanoic acid, and the uric acid/xanthine ratio were correlated with moderate CHD lesions, while the alpha-ketoisovaleric acid/valine ratio, gluconolactone, 4-hydroxycinnamic acid, and sorbose were correlated with severe CHD lesions. At present, there are few studies on the relationship between small-molecule metabolites and CHD severity. Next, we will discuss several differential metabolites that are associated with the CHD severity.

Threonine is an essential amino acid for humans. It must be obtained from food and plays an important role in macromolecule biosynthesis, the modulation of nutritional metabolism, and gut homeostasis [22, 23]. The multiple pathways of threonine catabolism provide carbon for biosynthesis and epigenetic regulation of biological macromolecules affecting the self-renewal of embryonic stem cells (ESC) [24]. Dong et al. [25] found that the concentration of threonine in cardiac tissue is higher in cyanotic than acyanotic congenital heart disease. Conversely, in the present study, we observed a decrease in threonine level in amniotic fluid in CHD with moderate lesions compared to CHD with mild lesions. We hypothesized that these contrasting results might reflect the differences in sample types included in our analysis and that of Dong et al. [25]; however, the underlying mechanism remains to be identified.

Ornithine is a non-essential amino acid generated from arginine. It is mostly involved in the urea cycle, and the abnormalities in its metabolism are implicated in gyrate atrophy, cancer, hyperornithinemia, and hyperammonemia [26]. Schlüter and collaborators demonstrated that hypoxia could induce an increase in arginase, as an early response to hypoxia, which catalyzes the formation of ornithine and urea [27]. According to their findings, hypoxia may lead to increased ornithine levels. Conversely, in the present study, we observed a significant decrease in ornithine levels in CHD with moderate and severe lesions than in CHD with mild lesions. Thus, we suspect that the response to hypoxia may depend on CHD severity: mild hypoxia may increase ornithine concentration, but severe hypoxia may have the opposite effect. However, other possible explanations cannot be excluded.

Gluconolactone, a naturally occurring glucose derivative and antioxidant, has cardioprotective effects in humans [28]. We found significantly higher gluconolactone levels in moderate and severe types of CHD than in mild CHD types. This increase may represent a self-protective mechanism against cardiac disease. Pentadecanoic acid is an odd-chain saturated fatty acid, mainly obtained through dairy products, and there is a negative correlation between circulating pentadecanoic acid and the risk of metabolic disease [29]. Research from Khaw *et al.* [30] indicated that pentadecanoic acid was negatively associated with the risk of coronary heart disease. In this study, we found that the level of pentadecanoic acid in moderate CHD lesions was significantly lower than that in mild lesions, with a Spearman's correlation coefficient of -0.454, which was consistent with the findings of Khaw et al. [30].

Sorbose is a monosaccharide that can be detected in human feces, blood, and urine, while 4-hydroxycinnamic acid, a derivate of cinnamic acid, is abundant in plant seeds and leaves [31–34]. At present, there are no reports on the relationship between sorbose, 4-hydroxycinnamic acid, and CHD severity.

In summary, untargeted metabolomics documented the absence of significant differences in the overall metabolic profile among CHD with mild, moderate, and severe lesions. However, there were some differences in the levels of metabolites among the different groups. The main shortcomings of this study are the relatively small sample sizes of each group and the lack of validation samples. If conditions permit, we will collect verification cohort samples for verification in the subsequent work. In addition, due to the lack of normal control samples matched for gestational age, the use of differential metabolites for distinguishing non-CHD requires further investigation. Despite this, this performed analysis study can enable us to clarify the overall metabolic status of CHD with different severity and provide novel concepts to be used in investigating the mechanism of CHD.

Declarations

Ethics approval and consent to participate

This study was approved by Medical Ethics Committee of Nanjing Maternity and Child Health Care Hospital (Code number: [2018] NO.91). All participants signed informed consent before undergoing amniocentesis, and agreed for the use of the remaining samples from clinical tests for scientific research. The need for written informed consent was waived by the Nanjing Maternity and Child Health Care Hospital ethics committee due to retrospective nature of the study. All methods were carried out in accordance with relevant guidelines and regulations under Ethics approval.

Consent for publication

Not applicable because there is no details, images, or videos related to an individual person.

Availability of data and materials

Data is available from the corresponding author Tao Jiang, and the email is jiangtao6310@126.com.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by National Natural Science Foundation of China (81770236), Jiangsu Natural Science Foundation (BK20181121), and the National Key Research and Development Program of China (2018YFC1002402).

Authors' contributions

YL and YS were responsible for comprehensive sample collection, data collection, experimental operation, data analysis, figure preparation, table preparation and paper writing. PY, XW and XZ were responsible for sample and information collection. ZX, TJ and PH were responsible for the research design, funding, and paper writing. All authors contributed to the article and approved the submitted version.

Acknowledgements

We thank all the participants for their contributions.

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Figures

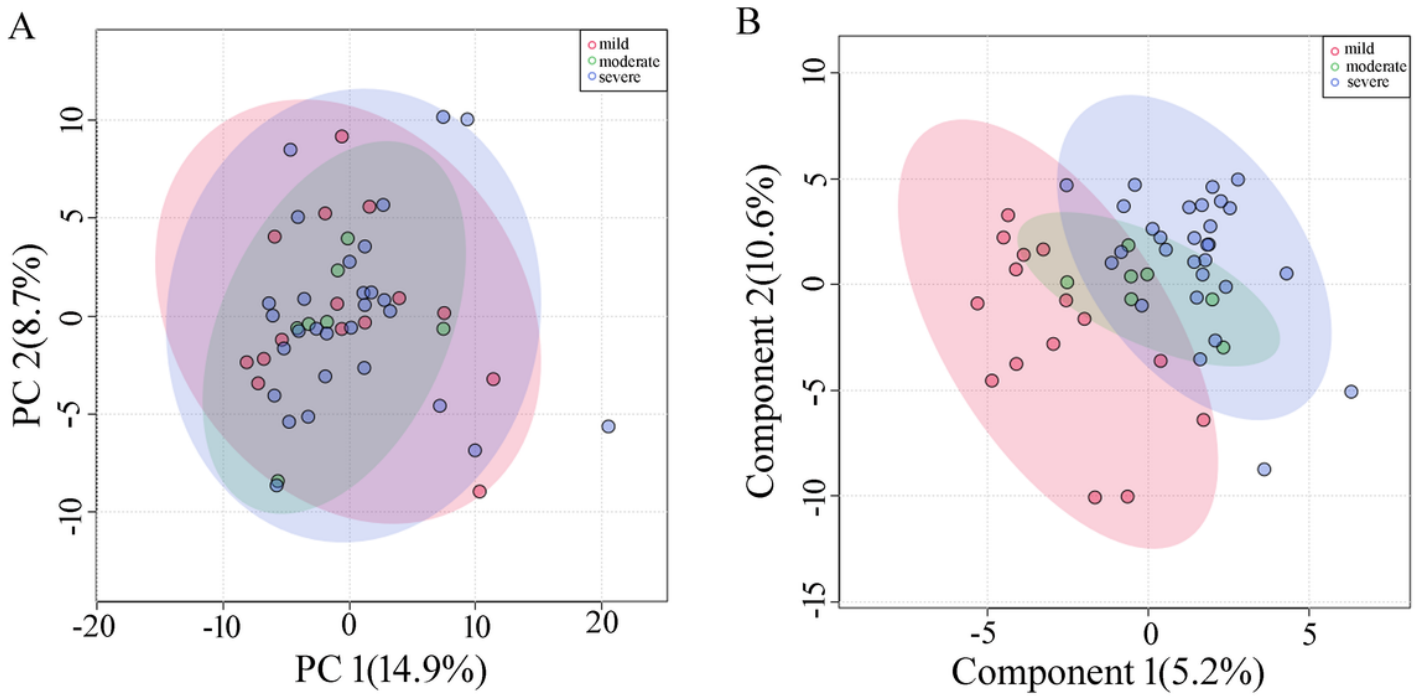

Figure 1

Metabolic profiles among CHD with mild, moderate, and severe lesions. (A) Unsupervised PCA model; (B) PLS-DA model, $R^2Y=0.90$, $Q^2= -0.36$.