Metabolomic profiling reveals amino acid dysregulation in congenital heart disease: Arginine-induced embryonic malformations and pathogenic insights

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Abstract. Congenital heart disease (CHD) is the most common form of malformation seen in China. In most cases, its pathogenesis is unclear. The present study aimed to identify the differential metabolites and novel screening markers to discover the potential pathogenesis of CHD. Cultured amniotic fluid cells from pregnant patients carrying CHD-affected (n=24) or healthy (n=24) fetuses were collected. Untargeted metabolomics was performed on the cells. A total of 292 metabolites (172 up- and 120 downregulated) were significantly different between the CHD and control groups (variable importance on projection >1 and P<0.05). Significantly different metabolites were screened and analyzed using hierarchical clustering and Kyoto Encyclopedia of Genes and Genomes metabolic pathway enrichment. These data demonstrated that amino acid metabolism was considerable elevated. The most notable differential metabolites were lysine and arginine, suggesting they may play an important role in heart development. When arginine were used to treat pregnant mice, embryos (embryonic day 15.5) demonstrated increased malformation rate, the litter size decreased (4.3±1.03 vs. 6.5±1.05, P<0.01) and the naked deformity rate significantly increased (30.8 vs. 0.0%, P<0.001), compared with the controls. The embryonal heart cavity was larger and the heart wall became thinner. The present study suggested that amino acid metabolites may serve a crucial role in heart development and could serve as potential screening markers for CHD. Additionally, the adverse effects of arginine treatment on embryonic development highlight its role in CHD pathogenesis.

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Introduction

In China, congenital heart disease (CHD) is the most prevalent type of birth defect, accounting for ~30% of all congenital birth defects in 2023 (1). It affects 17-32‰ perinatal births (1). The incidence of prenatal CHD is also increasing. CHD incidence in 2020 was 4.3 times that in 2010 (1). CHD is divided into non-syndromic and syndromic phenotypes. Approximately 1/3 cases have definite genetic or chromosomal abnormalities, of which ~23% of CHD cases have chromosomal aneuploidy or copy number variation, and ~10% have new mutations or genetic mutations inherited from their parents (2,3). The pathogenesis is unclear in most cases, especially in non-syndromic phenotypes. It is well accepted that CHD is attributed to the co-effects of genetics, epigenetics and environment (2-5). Nutritional imbalances, drinking, smoking, drugs, hypoxia and other environmental factors can increase the incidence of CHD (4,5). Prenatal diagnosis of CHD mainly relies on ultrasonography. Hence, it cannot be discovered until the defect develops to a stage that can be measured using ultrasound (6). Therefore, additional molecular markers are required for early screening.

Metabolites are the end products of all processes occurring in cells. Metabolomics provides methods for identifying changes in metabolite profiles to support early biomarker discovery, disease diagnosis and treatment. Metabolomics, which is performed on a high-throughput platform, refers to the screening, identification, quantification and characterization of biochemicals that are <1,800 Da and are involved in various biological pathways (7). Metabolomics represents a bridge between the genome and phenotype and serves a connecting role in the transmission of biological information. The disease or physiological state can be better reflected in the metabolomic profile than in the transcriptome or proteome profile (8). Effective small changes in gene and protein expression can be amplified by metabolites, making detection easier. Metabolomics has a simple metabolite information base,

which is less complex than others (genomics, transcriptomics and proteomics). Several metabolomics studies have focused on fetal CHD, using maternal serum and urine and amniotic fluid samples (7-9). To the best of our knowledge, amniotic fluid cells have not been used for metabonomic screening of CHD. The present study used cultured amniotic fluid cells from patients carrying fetuses with CHD for untargeted metabolomics screening to discover the potential pathogenesis and screen new molecular markers.

Materials and methods

Sample collection and preparation. The cultured amniotic fluid cells [healthy controls (no chromosomal or other disease), n=24; CHD cases (fetus with ventricular septal defects), n=24] were obtained from singleton pregnant patients who underwent amniocentesis during gestational weeks 20.0-26.3. The patients were aged from 22 to 43 years old. Inclusion criteria: Patients were willing to have amniocentesis. In CHD group, the fetus was diagnosed as CHD by ultrasound. The indication for amniocentesis in the control group was either older than 35 years, a high risk in serum Down's screening or a high risk of non-invasive prenatal screening. Exclusion criteria: The fetus was affected with abnormalities chromosomes or abnormal copy number variation. The samples were collected in Shengjing Hospital of China Medical University (Shenyang, China) from April 2022 to December 2022. All participants provided written informed consent, and the study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (approval no. 2022PS307K). The sample collection and experimental procedures conformed to the Declaration of Helsinki. All patients were non-smokers and non-alcoholics. The samples were matched for maternal and gestational age and sex of the fetus (Table I). The amniotic fluid cells were cultured with amniotic fluid medium (FUJIFILM Biosciences; cat. no. 99473210805) for 2 weeks in 5% CO_2 at 37˚C (Fig. 1).

The metabolites were extracted from the cell residue with 1 ml precooled methanol/acetonitrile/water (v/v, 2:2:1) under sonication every 10 min for 1 h in ice baths. The mixture was incubated at -20˚C for 1 h, followed by centrifugation at 14,000 x g at 4˚C for 20 min, and the supernatant was transferred to a sampling vial. To ensure data quality for metabolic profiling, quality control (QC) samples were prepared by pooling aliquots representative of all samples for data normalization. The supernatant was evaporated using a high-speed vacuum-concentration centrifuge, 14,000 x g and 4˚C about for 1 h. Dried extracts were redissolved in 50% acetonitrile. Each sample was centrifuged at 14,000 x g and 4˚C for 20 min, and the supernatant was used for analysis.

Ultra-high performance liquid chromatography (UHPLC)-tandem mass spectrometry (MS/MS) analysis. Metabolomic profiling was performed on UPLC-MS/MS system (1290 Infinity LC, Agilent Technologies, Inc.) and TripleTOF 5600 (SCIEX). Then, 5 μl Samples were separated with a 2.1x100.0 mm ACQUIY UPLC BEH 1.7 μM column (Waters China Ltd.). The flow rate was 0.5 ml/min and the mobile phase contained A (25 mM each ammonium acetate and ammonium hydroxide in water) and B (acetonitrile). The gradient was 95% B for 0.5 min and was linearly reduced to 65% in 6.5 min, 40% in 2.0 min (maintained for 1.0 min) and increased to 95% for 1.1 min, employing a 5-min re-equilibration period. Both electrospray ionization (ESI) positive and negative modes were applied for MS data acquisition. The ESI source conditions were as follows: Ion source gas 1 and 2, 60; curtain gas, 30; source temperature, 600˚C; ion spray voltage floating, ±5,500 V. In MS acquisition, the instrument was set to acquire data over the m/z range of 60-1,200 Da, and the accumulation time for MS scanning was 0.15 sec/spectra. In the auto MS/MS acquisition, the instrument was set to acquire data over the m/z range of 25-1,200 Da, and the accumulation time for the production scan was 0.03 sec/spectra. Blank (50% acetonitrile in water) and QC samples were injected every 12 samples during acquisition. All the samples were run in one batch for the positive and negative ion mode. Each sample was detected and analyzed using UPLC-MS/MS, and two original files (positive and negative ion mode) were obtained. MS total ion chromatogram (TIC) flow diagram was generated.

Data preprocessing and filtering. Data processing and analysis were as previously described with minor amendments (10). Raw MS data were converted to MzXML files using ProteoWizard MS Convert and processed using XCMS for feature detection, retention time correction and alignment. The metabolites were identified by accurate MS (<25 ppm) and MS/MS data, which were matched with a standard database (BaseDeepBP, Shanghai Bioprofile Technology, Human Metabolome Database (hmdb.ca/, MassBank, https://massbank.eu/MassBank/, MetaboBASE, http://metabase.com/, Global Natural Product Social Molecular Networking, https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash-old.jsp). In the extracted-ion features, only the variables with >50% of the non-zero measurement values in ≥1 group were retained.

Multivariate statistical analyses. All multivariate data analyses and modeling were conducted using the SIMCAP software (Version 14.0, Umetrics; Sartorius AG). After mean-centering the data using Pareto scaling, models were developed using principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA) and PLS-DA. All models were evaluated for overfitting using permutation tests. Descriptive performance was assessed using cumulative R2X [ideal model: R2X (cumulative)=1 and R2Y (ideal model: R2Y (cumulative)=1] values, while prediction performance was measured using cumulative Q2 [ideal model: Q2 (cumulative)=1)] and a permutation test. In the permuted model, R2 and Q2 values at the Y-axis intercept should be lower than those of the non-permuted model. OPLS-DA was used to identify discriminating metabolites using variable importance on projection (VIP) scores, which indicate a variable contribution to class discrimination. VIP scores were calculated as the weighted sum of squares of the PLS weights, with values >1 considered statistically significant. High VIP scores indicate strong discriminatory ability and help in selecting biomarkers. Discriminating metabolites were obtained using a statistically significant threshold of VIP values from the OPLS-DA model and two-tailed Student's t-test (P-value) on normalized raw data. Metabolites with VIP

Table I. Clinical information for amniotic fluid cells.

Group	n	Mean maternal age, years	Mean gestational age, weeks	Fetus sex (male/female)
Control	24	31.6±5.45	22.4±1.88	11/13
CHD	24	30.9±4.65	25.0±1.35	12/12

CHD, congenital heart disease.

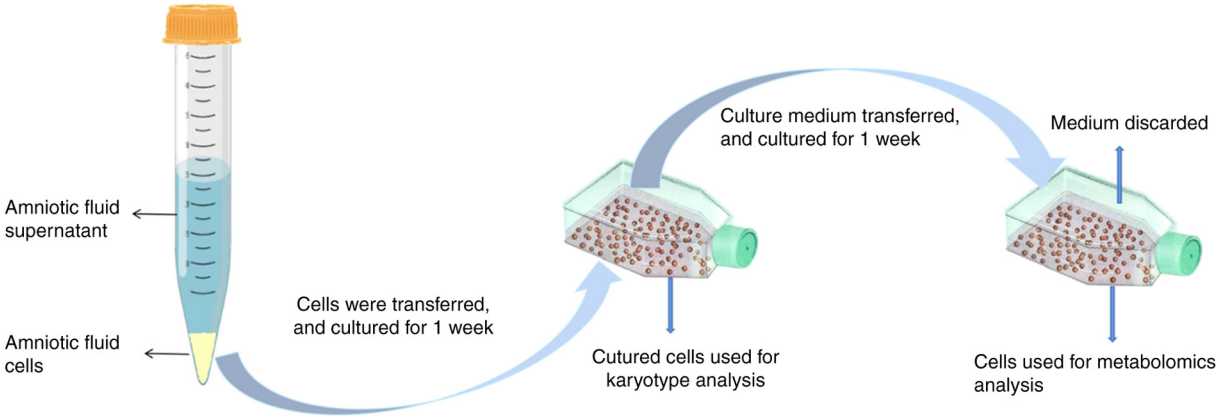

Figure 1. Experimental workflow. Amniotic fluid cells were obtained by centrifugation and cultured for 1 week for karyotype analysis. The culture medium transferred and cultured for 1 week. Cells cultured for 2 weeks that would otherwise be discarded were used for metabolomic analysis.

values >1 and P<0.05 were considered statistically significant. The fold-change was calculated as the ratio of the average mass area of the CHD group to that of the control group. Identified differential metabolites were used for cluster analyses using the R package (R package version 1.0.12, CRAN.R-project.org/package=pheatmap). Hierarchical clustering for each group was performed for heatmap.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. To identify perturbed biological pathways, differential metabolite data were subjected to KEGG pathway analysis using the KEGG database (kegg.jp/). KEGG enrichment analyses were performed using the Fisher's exact test and false discovery rate correction for multiple testing was also performed.

Animals and arginine/lysine administration. A total of 18 Female C57BL/6J mice (age, 8-10 weeks, Shenyang Lan Pudas technology co., ltd) were used (mean weight, 22.2±0.81 g; range, 20.8-23.6 g). The mice were kept in standard cages under a 12/12-h light/dark cycle, *ad libitum* food and water, temperature was 20-26˚C and humidity was 40-70%. To obtain pregnant mice, female mice mated with male mice (n=6, 8-10 weeks age, 23.2-25.6 g weight, Shenyang Lan Pudas Technology co., ltd) and underwent testing every morning. Females with a vaginal plug were immediately separated from the males and indicated as embryonic day 0.5 (E0.5). Pregnant mice were randomly assigned to treatment (either arginine or lysine, Sigma) and control group (both n=6). The treatment group received a single intraperitoneal injection of 1.5 mg/g body weight/day arginine/lysine from E7.5 to E14.5. The control group received an equivalent volume of saline. Doses of arginine or lysine administration were determined as previously described (11-13). In addition to the routine feeding, mouse health and behavior were checked every 12 h. The humane endpoints were cessation of eating and movement and listlessness or lack of response when people approached. No animals reached these endpoints. Pregnant mice at E15.5 were anaesthetized with 1.5% isoflurane for 2-3 min. The anesthetized mice had even heartbeat and breathing, relaxed muscles and no limb activity or pedal reflex. A total of 100 μl peripheral blood was collected from the inner canthus by capillary tube. Then, mice were euthanized by cervical dislocation. Death was confirmed when heartbeat and respiration stopped and the pupils dilated for >5 min. Maternal plasma arginine concentration was determined by LC-MS/MS. All experiments were approved by the Ethics Committee of Shengjing Hospital of China Medical University (approval no. 2024PS202K).

Hematoxylin-eosin staining of embryonic heart. The embryonic trunk was fixed in 4% paraformaldehyde solution at 4˚C for ≥48 h. The samples were dehydrated in 75, 85, 95 and 100% ethanol for 1 h. Following xylene treatment at room temperature, 100% paraffin was used for tissue embedding. Then, continuous slices of the embryonic heart (4 μm) were cut from the top of the aortic arch to the apex of the heart and stained with hematoxylin and eosin at room temperature for 1 min using automatic stainer (Leica GmbH) and images were captured under a light microscope.

Detection of amino acids in maternal peripheral blood. Maternal peripheral blood (96 controls and 69 CHD cases;

gestational age, weeks 18.0-30.0, aged from 20 to 44 years old, collected in Shengjing Hospital of China Medical University from April 2022 to December 2022, the same Inclusion/exclusion criteria as amniotic fluid cells collection) were collected to detect the change of amino acids using a non-derivatized amino acids detection kit (Neobase 2, Revvity, Inc.) on LC-MS/MS (QSight 210MD, PerkinElmer). The sample was detected with multiple reaction monitoring mode scanning in positive ion mode. The ionization conditions were as follows: Drying gas, 120 l/min; electrospray voltage, 4,900 V; source temperature, 150˚C; nebulizer gas, 220 l/min; hot surface-induced desolvation temperature, 250˚C. The injection volume was 10 μl. Chromatographic mobile phase A (included in the detection kit) and the chromatographic gradient elution procedure was as follows: 0.00-0.15 min, flow rate, 0.16 ml/min; 0.15-0.90 min, flow rate, 0.03 ml/min; 0.90-1.00 min, flow rate, 0.7 ml/min and 1.0-1.15 min, flow rate, 0.16 ml/min. The parameters of amino acid detection MS are listed in Table SI.

Statistical analysis. All data are presented as the mean ± standard deviation. At least three independent experimental repeats. The analysis was performed using SPSS 17.0 software (SPSS, Inc.). Student's unpaired two-tailed t test, one-way ANOVA followed by least significant difference post hoc test and χ^2 test were used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Experimental quality evaluation. Metabonomics analysis was performed on 48 samples (24 controls and 24 CHD cases). Each sample was detected and analyzed using UPLC-MS/MS, and two original files (positive and negative ion mode) were obtained. Representative images are shown in Fig. 2A. A total of 883 metabolites were identified (data not shown). The system stability was evaluated using two strategies: MS total ion chromatogram (TIC) flow diagram comparison of QC samples and PCA statistical analysis of overall samples. A total of four QC TIC diagrams were overlaid (Fig. 2B). The results suggested that the response intensity and retention time of each color spectrum peak overlapped, indicating that the variation caused by instrument error was small and the data quality was reliable. The ion peaks of metabolites extracted from all experimental and QC samples were subjected to PCA. Samples were closely gathered together, indicating that the experiment had good repeatability (Fig. 2C). Thus, the instrument analysis system had good stability, and the experimental data were reliable. The metabolic spectrum difference reflected the biological differences between the samples.

OPLS-DA. An OPLS-DA model was established to compare metabolite profiles between the two groups. The model evaluation parameters and score diagram are shown in Fig. 3. The OPLS-DA model distinguished the two sample groups. The parameters (R2Y=0.925 and Q2=0.76) in the OPLS-DA model suggested that the experimental data were stable and reliable. The intercept of Q2 was -0.319, indicating there was no overfitting in the OPLS-DA model established using the experimental data. The OPLS-DA model showed separation in the metabolic profiles between the CHD and control groups. A total of 292 different metabolites (VIP>1 and P<0.05) were identified, of which 172 metabolites were up- and 120 metabolites were downregulated in the CHD compared with the control group (supplementary data). The two most changed metabolites were lysine (L-lysine/lysine/N-methyllysine) and arginine (homoarginine/L-arginine; Table II).

Univariate statistical analyses. To identify potential marker metabolites, univariate analysis was performed (FC<0.5 or >2.0). Lysine and arginine were the two most changed metabolites (Fig. 4). The fold-changes of L-lysine, lysine, homoarginine and L-arginine were 1848.4, 629.9, 766.5 and 38.2, respectively.

Hierarchical cluster analysis. To evaluate the potential differential metabolites, hierarchical clustering for each group was performed (Fig. S1). Generally, when the candidate metabolites screened are reasonable and accurate, the same group of samples appear in the same cluster. Metabolites in the same cluster have similar expression patterns. Although some samples strayed from their groups, most were concentrated in their respective groups.

KEGG pathway enrichment analysis. The differential metabolites were analyzed using KEGG (Fig. 5; supplementary data). The results demonstrated that amino acid metabolism (involved in 'ABC transporters', 'biosynthesis of amino acids' and 'D-arginine and D-ornithine metabolism') may play an important role in CHD development.

Arginine results in heart malformation. To test whether arginine/lysine affect heart development, arginine/lysine was intraperitoneal injected in pregnant mice. Litter size decreased, and the naked deformity rate increased after arginine treatment (Fig. 6A; Table III). The concentration of maternal plasma arginine increased following arginine treatment (182.6±14.4 vs. 92.3±9.6 μmol/l, Fig. 6B). In the arginine-treated group, the hearts of the embryos with no notable gross abnormality demonstrated enlarged heart cavities and thinner heart walls (Fig. 6C), compared with the control. In the lysine-treated group, no obvious deformity was observed.

Amino acids in maternal peripheral blood are not significantly altered between CHD and control group. As there were several amino acids changes in amniotic fluid cells metabonomics, the present study investigated whether the amino acids change in human maternal peripheral blood between CHD and control group. Compared with control group, glycine and glutamine were downregulated by 17.3% (503.8±147.1 vs. 608.7±266.8 μmol/l, Table IV) and upregulated by 6.7% (381.0±70.1 vs. 357.8±77.30 μmol/l), respectively. Therefore, these amino acids cannot be used as non-invasive markers for CHD screening.

Discussion

Samples from fetuses with CHD had different metabolic profiles compared with controls. It was determined that amino acids serve an important role in heart development. When arginine was used to treat the pregnant mice, the embryos

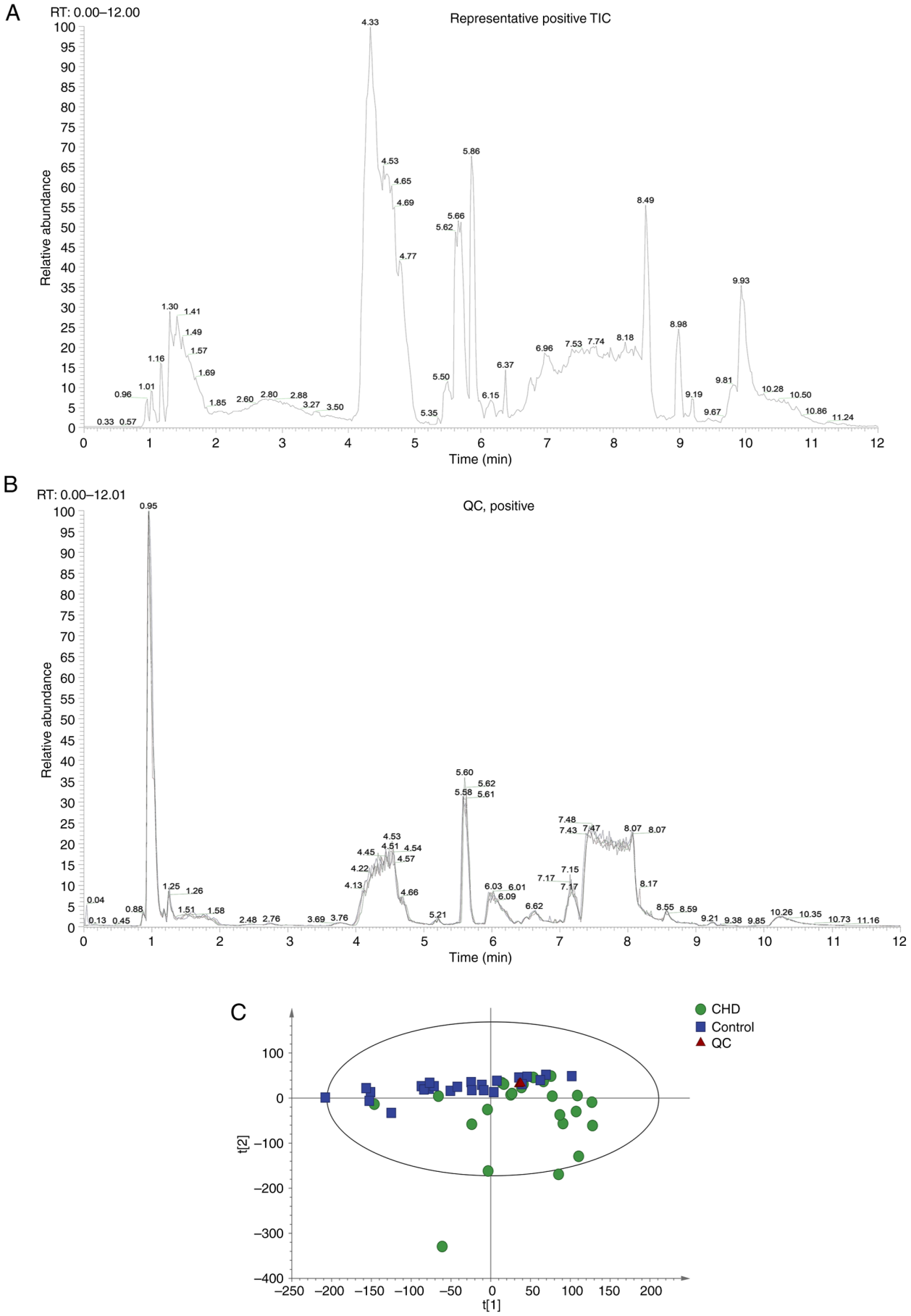

Figure 2. Representative metabolic profile and experimental quality evaluation. (A) Representative TIC from sample in electrospray ionization positive mode. (B) TIC overlapping map of positive modes of quality control sample. (C) Principal component analysis score chart of samples. TIC, total ion chromatogram; QC, quality control; CHD, congenital heart disease; RT, retention time.

Table II. Differential metabolites in congenital heart disease compared with controls.

Metabolite	ESI mode	RT, min	m/z	VIP	Fold-change	P-value
L-lysine	Negative	9.954	145.097	1.67	1,848.38	0.0005
Lysine	Positive	10.013	147.112	1.41	629.88	0.0042
N-methyllysine	Negative	9.886	159.112	1.76	50.56	0.0002
Homoarginine	Negative	9.853	187.119	1.74	766.51	0.0003
L-arginine	Positive	10.086	175.119	1.58	38.19	0.0015
D-aspartate	Negative	8.672	132.029	1.27	25.97	0.0034
L-asparagine	Negative	8.445	131.045	1.24	7.26	0.0049
Proline	Positive	10.139	116.071	1.51	22.71	0.0023
L-ornithine	Negative	10.061	131.081	1.49	11.66	0.0016
Glutamine	Negative	8.200	145.061	1.33	11.28	0.0038

ESI, electrospray ionization; RT, retention time; VIP, variable importance on projection.

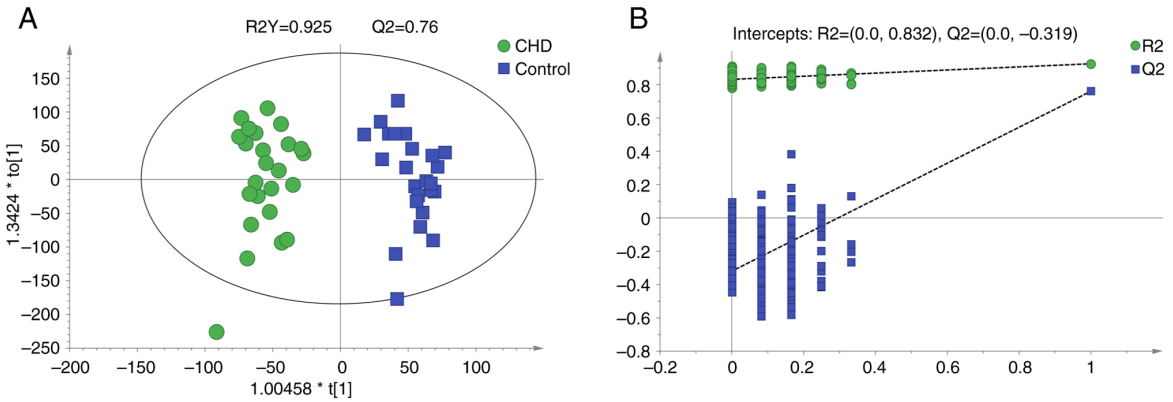

Figure 3. Significant differential metabolite analysis. (A) OPLS-DA between control and CHD groups. The values of R2Y and Q2 demonstrated the goodness of fit and predictability of the model, respectively. (B) Permutation test of the OPLS-DA model. The intercept of Q2 suggested no overfitting in the model. OPLS-DA, orthogonal partial least squares discriminant analysis; CHD, congenital heart diseas.

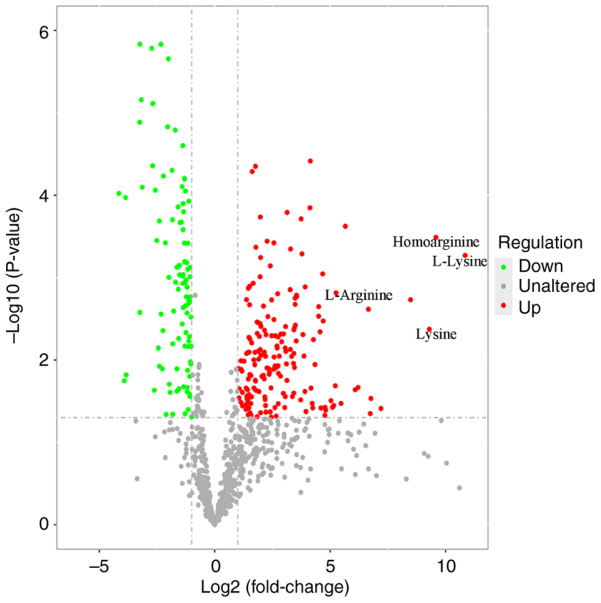

Figure 4. Volcano plot of CHD vs. control. Association between fold-change of CHD vs. control and significance of the metabolic features. Upregulated, fold-change >2 and P<0.05; downregulated, fold-change <0.5 and P<0.05. CHD, congenital heart diseas.

demonstrated increased malformation rate. The embryonal heart cavity became larger and the heart wall became thinner. The present study may provide insight into the pathophysiology of CHD.

The high incidence of CHD have prompted investigation into its pathogenesis to identify early screening indicators (1). With the rapid development of high-throughput sequencing technology, an increasing number of copy number variations and gene mutations have been identified in CHD (2,3). However, only one-third of the cases can be explained by genetics (2,3). The wide application of metabolomics facilitates investigation of potential CHD pathogenesis or early screening biomarker.

There are three primary metabolomics detection platforms, namely LC-MS, gas chromatography-MS (GC-MS) and nuclear magnetic resonance (NMR) (14,15). Because NMR has defects, such as relatively low sensitivity, poor selectivity and limited metabolite coverage, MS platforms with high sensitivity and specificity are more commonly used (14-16). Compared with GC-MS, LC-MS has advantages: The samples are separated at room temperature without special treatment (GC-MS often requires derivatization). In addition, the range of the molecular weights of the tested substances is wider. Moreover, it has

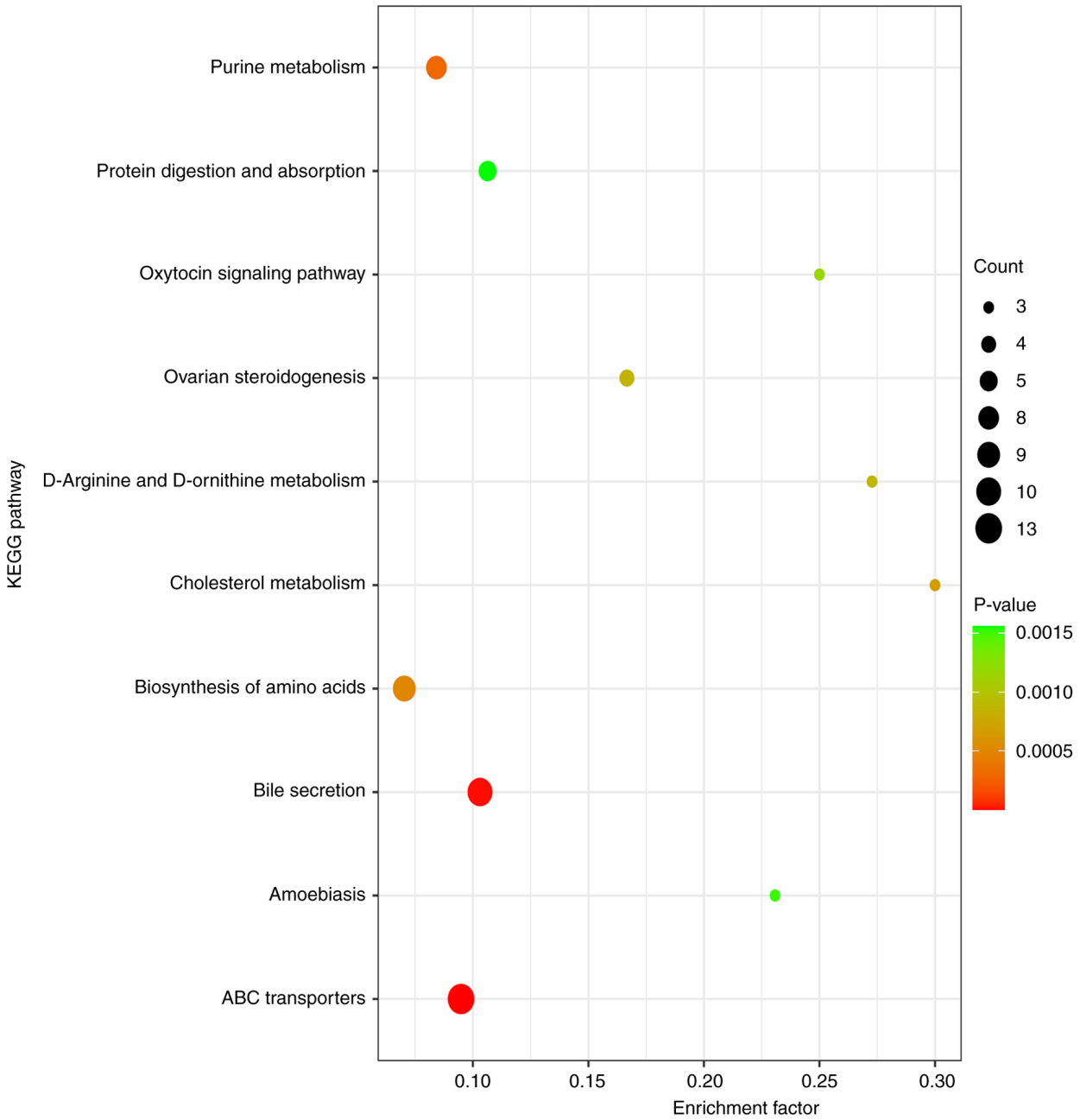

Figure 5. KEGG pathway enrichment analysis of the differential metabolites. Amino acids (L-lysine, -arginine, -glutamine, -ornithine and -asparagine) were involved in 'ABC transporters', 'biosynthesis of amino acids', 'purine metabolism', 'protein digestion and absorption', 'D-arginine and D-ornithine metabolism' and 'amoebiasis'. KEGG, Kyoto Encyclopedia of Genes and Genomes; ABC, ATP-binding cassette.

higher sensitivity and can detect more trace substances. Thus, LC-MS has a wide range of applications (14-16). Several types of biological samples (amniotic fluid, plasma, urine) have been used for metabolomics screening (9). The present study used cells; as the basic functional unit of life, cells directly reflect the levels of intracellular metabolism and are more conducive to discover the potential pathogenic mechanisms.

To explore the possibility of arginine and other amino acids as metabolic markers, the present study detected the concentration of amino acids in maternal peripheral blood. There was no significant difference between CHD and control group, indicating plasma amino acids cannot be used as non-invasive markers for CHD screening. Although the present study demonstrated a number of differential metabolites in amniotic fluid cell metabonomics, there were limitations. First, the present study could not determine whether the altered metabolites were the result or the cause of CHD. More studies are needed to clarify this. Second, the sample size was not sufficiently large, and the type of CHD was only concentrated in the ventricular septal defect. A comprehensive, large-scale multi-center study is needed.

L-lysine, an essential amino acid, was the most notable differential metabolite identified in CHD. Humans receive L-lysine from daily food such as meat, cereal grains or legumes (17). L-lysine is taken up into cells by cationic transport system y+ (18). Lysine has important functions in the promotion of human physiological development and fatty acid oxidation (19). It promotes brain development and fat metabolism

Table III. Effects of arginine/lysine treatment on mice.

Group	Pregnant mice, n	Embryos, n	Mean litter size	Deformity rate[a], % (n)
Control	6	39	6.5±1.05	0 (0)
Arginine	6	26	4.3±1.03[b]	30.8 (8)[c]
Lysine	6	36	6.0±1.41	0 (0)

[a]Abnormal embryos visible to the naked eye. [b]P<0.01, [c]P<0.0001 vs. control.

Table IV. Mean concentration of amino acids in maternal peripheral blood.

Amino acid	Control (n=96), μM	CHD (n=69), μM	T-value	P-value
Ala	504.3±142.0	472.9±127.2	1.489	0.138
Val	177.7±34.7	176.6±33.8	0.217	0.828
Gly	608.7±266.8	503.8±147.1	3.230	0.002
Gln	357.8±77.3	381.0±70.1	-2.011	0.046
Glu	159.1±48.9	144.7±54.6	1.740	0.084
Orn	106.5±18.9	105.6±17.1	0.314	0.754
Leu	181.0±40.7	174.1±43.9	1.013	0.313
Arg	11.8±3.9	12.4±4.8	-0.75	0.455
Met	13.4±5.2	13.3±5.5	0.041	0.968
Phe	73.1±26.4	72.2±19.5	0.248	0.804
Tyr	57.5±12.6	54.7±15.7	1.220	0.225
Cit	16.8±3.2	17.4±4.5	-0.913	0.363
Pro	145.2±37.6	135.9±33.0	1.692	0.093
HArg	11.4±3.1	11.2±2.8	0.518	0.605
Lys	49.5±9.5	49.9±7.8	-0.289	0.773

HArg, homoarginine; CHD, congenital heart disease.

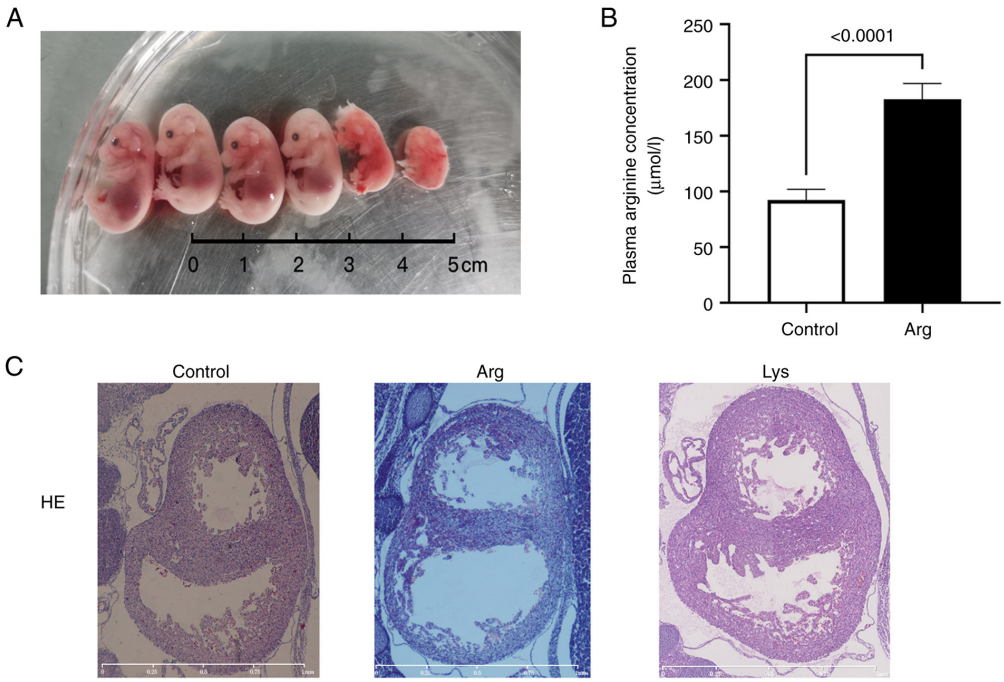

Figure 6. Arg results in heart malformation but Lys does not induce notable abnormality. (A) Abnormal embryos were found in Arg-treated group. (B) Maternal plasma Arg concentration following Arg treatment. (C) HE staining suggested embryos had enlarged heart cavities and thinner heart walls following Arg treatment. HE, hematoxylin-eosin.

and prevents cell degeneration. Shimomura *et al* (20) found that dietary L-lysine exerts protective effects against vascular calcification in uremic rats. The addition of lysine to the diet can manage osteoporosis (21). L-lysine also has beneficial hemodynamic effects and decreases nitric oxide levels in endotoxemic rats (22). Lysine acetylsalicylate can recover sepsis-induced lung tissue damage in rats (23), and L-lysine suppresses acute pancreatitis in mice (24). L-lysine ameliorates sepsis-induced acute lung injury in a lipopolysaccharide-induced mouse model (25). Abnormalities in lysine degradation are involved in the early development of cardiomyocyte hypertrophy in pressure-overloaded rats (19). In the present study, lysine did not cause any difference in the litter size and deformity rate from the controls and embryonal hearts also did not show obvious malformation (the data not shown in this study). Although lysine has a range of functions, more studies are needed to clarify its role in heart development.

L-arginine, homoarginine, L-ornithine, L-asparagine and L-glutamine belong to the arginine family and participate in the urea cycle (26). Glutamine, glutamate and arginine can regulate nutrient intake and neonatal development through norepinephrine, glucagon-like peptide-1, mammalian target of rapamycin, mitogen-activated protein kinase and autophagy (26,27). Arginine is an important nutrient with regulatory roles in neonatal growth (28,29). Rat studies have indicated that supplementing the maternal diet with arginine and glutamine improves embryo implantation and survival, enhances litter size and increases the number and birth weight of surviving embryos (30,31). In addition, arginine and glutamine affect gene expression to improve antioxidant responses and DNA transcription (32,33). Plasma homoarginine levels have been reported to show a declining trend in patients with complex CHD compared with healthy controls (34). Therefore, homoarginine may be a prognostic indicator of CHD. Cedars found that plasma concentrations of multiple amino acids differ between adult patients with CHD and healthy controls (35). Although the numerical difference is not large, a metabolite cluster containing amino acids and metabolites is associated with negative clinical outcomes (35). A total of 11 amino acids (including arginine, lysine, asparagine and glutamine) are upregulated in the cardiac tissues of children with cyanotic CHD (36) compared with those with acyanotic CHD. Yu *et al* (37) found that glutamine and glutamate have considerable diagnostic value for pediatric patients with CHD patients. Li *et al* (9) found that the concentrations of uric acid and proline in the amniotic fluid are significantly elevated in patients with CHD. In the present study, when pregnant mice were treated with arginine at the dose of 1.5 mg/g body weight, the litter size decreased and the embryo deformity rate increased. The embryonal hearts demonstrated enlarged heart cavities and thinner heart walls. The present study only tested the maternal plasma arginine concentration following arginine treatment and did not test the concentration in the placenta and heart in the embryo. Thus, changes in amino acid levels may serve an important role in the occurrence and development of CHD. Future studies should focus on metabolic reprogramming to clarify the potential mechanism of arginine-induced cardiac malformation.

In summary, differential metabolites were identified in CHD using metabolomics. The levels of numerous types of amino acids were significantly elevated, indicating that they may serve an important role in heart development. Arginine treatment of pregnant mice resulted in embryonal heart defects.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZL collected the samples and wrote the manuscript. CC performed animal experiments. JL analyzed the data. GL interpreted the data, performed experiments and revised the manuscript. CQ designed the study and revised the manuscript. ZL and GL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (approval nos. 2022PS307K and 2024PS202K). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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