



The value of lipid metabolites 9,10-DOA and 11,12-EET in prenatal diagnosis of fetal heart defects



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ABSTRACT

Aims: To explore the maternal metabolic changes of fetal congenital heart disease (FCHD), and screen metabolic markers to establish a practical diagnostic model.

Methods: Maternal peripheral serum from 17 FCHD and 63 non-FCHD pregnant were analyzed by Ultra High-performance Liquid Chromatography-Mass/Mass (UPLC-MS/MS).

Results: In the FCHD and the non-FCHD, 132 metabolites were identified, including 35 differential metabolites enriched in the purine, caffeine, primary bile acid, and arachidonic acid metabolism pathway. Finally, the screened (+/-)-9,10-dihydroxy-12Z-octadecenoic acid (AUC = 0.888) and 11,12-epoxy-(5Z,8Z,11Z)-icosatrienoic acid (AUC = 0.995) were incorporated into the logistic regression model. The AUC value of the two-metabolite model was 1.0, superior to proline (AUC = 0.867), uric acid (AUC = 0.789), glutamine (AUC = 0.705), and taurine (AUC = 0.923) previously reported. The clinical decision curve analysis (DCA) showed the highest clinical net benefit of the model, and internal validation by bootstrap shows the robustness of the model (Brier Score = 0.005).

Conclusion: For the prenatal diagnosis of CHD, our findings are of great clinical significance. As an additional screening procedure, the identification model might be used to detect.

1. Background

CHD is the most common congenital abnormality and one of the major causes of neonatal death, affecting about 0.4–1.0 % of children (<18 years old) [1]. Although there are many types of CHD, only a few types of CHD recover naturally, and some develop more and more complications as they age. With short life expectancy [2], and poor quality of life [3], CHD children bring a heavy burden on families and society [4]. Early intervention in some types of CHD after birth may

improve neonatal outcomes, including survival and neurocognitive outcomes [5]. Prenatal diagnosis is an important way to find out the abnormality of cardiovascular system in time, which is helpful to get timely treatment after birth. Additionally, through prenatal screening, termination of pregnancy is an effective means to reduce the incidence of CHD in children [6,7]. Prenatal screening of CHD relies heavily on echocardiography which still faces several practical problems, including the possibility of missed diagnosis, the influence of fetal position, the susceptibility to subjective judgment, the need for experienced

Abbreviations: 11,12-EET, 11,12-epoxy-(5Z,8Z,11Z)-icosatrienoic acid; 9,10-DOA, (+/-)-9,10-dihydroxy-12Z-octadecenoic acid; AUC, Area under the curve; CHD, Congenital heart disease; DCA, Decision curve analysis; FC, Fold change; FCHD, Fetal congenital heart disease; FDR, False Discovery Rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, Liquid Chromatograph Mass Spectrometer; OPLS-DA, Orthogonal partial least squares-discriminant analysis; PCA, Principal components analysis; QC, Quality control; ROC, Receiver operating characteristic; SD, Standard deviation; UPLC-MS/MS, Ultra High-performance Liquid Chromatography-Mass/Mass.

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diagnostic ultrasound specialists, and the availability of equipment in poor areas [8]. It is an urgent problem to establish a low-cost, rapid, and efficient screening method for prenatal diagnosis of congenital heart defects.

Previously, Wagner *et al.* [9] reviewed prenatal maternal diagnostic markers for congenital anomalies, including four cytoskeleton proteins, two miRNA, and five lncRNA as potential markers for CHD diagnosis. Not only the cost of detecting RNA is high, but miRNA and lncRNA are unstable molecules, and the sample preservation is cumbersome. It is unclear whether these markers can be used in clinical trials, and the testing may be expensive and time-consuming. Therefore, there is an urgent need for a more effective and cheaper FCHD screening method. Metabolomics based on Liquid Chromatograph Mass Spectrometer (LC-MS) has the characteristics of high throughput and sensitivity. In addition, a growing number of studies have explored metabolomics as one of the mainstream approaches for biomarkers, including cardiovascular diseases and cancers [10,11]. Furthermore, specific metabolites in maternal body fluids have been verified to predict birth outcomes (preterm, small for gestational age, large for gestational age) [12].

Up to now, several studies have focused on metabolic markers of fetal heart malformations. A Britain cohort study conducted by Bahado-Singh *et al.* [13] found that the combination of three lipid molecules had potential value in predicting fetal CHD (FCHD). In addition, a metabolomics study in the Italian population identified nine FCHD-related markers and established a machine learning model [14]. Recently, metabolic changes in amniotic fluid or urine in pregnant women with FCHD have also received special attention and potential markers have been identified [15,16]. Currently, maternal serum metabolic markers or models of FCHD in the Chinese population are blank.

Here, based on the analysis of maternal serum during pregnancy by LC-MS, the characteristics of maternal serum metabolism of FCHD were revealed. Importantly, we established a practical metabolic model based on two lipid metabolites for diagnosing FCHD.

2. Materials and methods

2.1. Study subjects and sample collection

This study is a single-center, observational study from September 2020 to May 2021. Pregnant women, 20–40 years old and singleton pregnant, were recruited in the Maternal and Child Health Hospital of Hubei province, including 20 FCHDs and 94 non-FCHDs. FCHDs were determined by echocardiography. History of infectious diseases, chronic diseases (hypertension, diabetes, and kidney diseases), and multiple malformations were excluded. Pregnant women in the non-FCHD group were followed to the end of delivery to ensure no obvious abnormalities. Demographic characteristics data were captured in the medical records system. The centrifuged peripheral serum was obtained from the clinical laboratory, then stored at –80 °C. The study flow diagram is shown in Fig. S1. This study has been reviewed and approved by the Medical Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (2019S065). All participants in the study received informed consent.

2.2. Sample pretreatment

Take out the serum previously stored at –80 °C and thawed it on ice. Then, 400 μL precooled methanol/acetonitrile (1:1, v/v) solution (Dikma Science and Technology Co. Ltd, Canada) was added to 100 μL serum, vortex for 30 s, centrifuged at 14000 rpm for 10 min at 4 °C. The 400 μL supernatant was then transferred to a clean tube, dried with nitrogen gas at room temperature, dissolved in 100 μL water/acetonitrile (95:5, v/v) solution, centrifuged at 4 °C for 10 min with a vortex of 30 s, 14000 rpm, and transferred to LC vials. The quality control (QC) samples were prepared by pooling aliquots (10 μL) of each sample.

2.3. LC–MS analysis and data processing

The supernatant was analyzed by UPLC-Q-Exactive-MS/MS (Thermo, USA). The LC-MS analysis process referred to Shen *et al.* [17] and Chen *et al.* [18] made some modifications. Details of LC-MS analysis and data processing can be found in the Supplementary Information.

2.4. Statistical analyses

The categorical data are described as frequency counts and percentages, and the continuous variables are presented as mean (\pm standard deviation [SD]) or median (first quartile [Q1] – third quartile [Q3]).

Before further analysis, metabolic intensities were \log_2 -transformed. Principal components analysis (PCA), orthogonal partial least squares-discriminant analysis (OPLS-DA), cluster heatmaps, volcano maps, correlation heatmaps, network analysis, and KEGG enrichment analysis were implemented by MetaboAnalyst 5.0 [19] and R software (version 4.1.2). The variables of differential metabolites were screened by lasso regression, and the screened variables were included in the model. We further performed a stepwise logistic regression analysis to determine the best subset of potential biomarkers screened by lasso regression. Here, we established a diagnostic model of FCHD based on logistic regression. The discrimination was assessed using the receiver operating characteristic (ROC) curve, and the clinical utilities were assessed using the clinical decision curve analysis (DCA). We also conducted internal verification of the model through the bootstrap method to ensure the stability of the model. We performed the statistical modeling using.

3. Results

3.1. Demographics, birth and clinical characteristics of the subjects

The CHD types for each case are detailed in Table S1. Demographics and birth characteristics are shown in Table 1. Maternal age of FCHD and non-FCHD ranged from 21 to 34 years (mean, 27.8) and 21 to 39 years (mean, 29.3), respectively. At the time of sample collection, the mean gestational age was 173 days in the FCHD group and 178 days in the non-FCHD group.

3.2. Metabolomic Profiling of FCHD maternal serum samples

In total, 132 compounds were identified. We examined the data globally with PCA and OPLS-DA, indicating the FCHD and non-FCHD samples were well distinguished (Fig. 1a, b and, Fig. S2a). The levels of 80 metabolites between the two groups changed significantly (60.61

Table 1
Demographics and Birth Characteristics.

	Overall	FCHD	non-FCHD
Number of samples	N = 80	N = 17	N = 63
Maternal age, years	28.9 ± 3.8	27.8 ± 3.4	29.3 ± 3.8
Previous births, No. (%)			
0	52 (65.00)	11 (64.71)	41 (65.08)
1	21 (26.25)	5 (29.41)	16 (25.40)
≥2	2 (2.50)	1 (5.88)	1 (1.59)
Missing	0 (0)	0 (0)	5 (7.94)
Times of pregnancies, No. (%)			
1	41 (51.25)	9 (52.94)	32 (50.79)
≥2	34 (42.5)	8 (47.06)	26 (41.27)
Missing	5 (6.25)	0 (0)	5 (7.94)
Gestational age at sample, days	177 ± 17	173 ± 29	178 ± 12
Pre-pregnancy BMI, kg/m ²	20.59 ± 4.19	20.29 ± 2.33	20.71 ± 4.74

Values are means (SDs) or numbers (Percentages).

FCHD: fetal congenital heart disease; BMI: body mass index; SD: standard deviation.

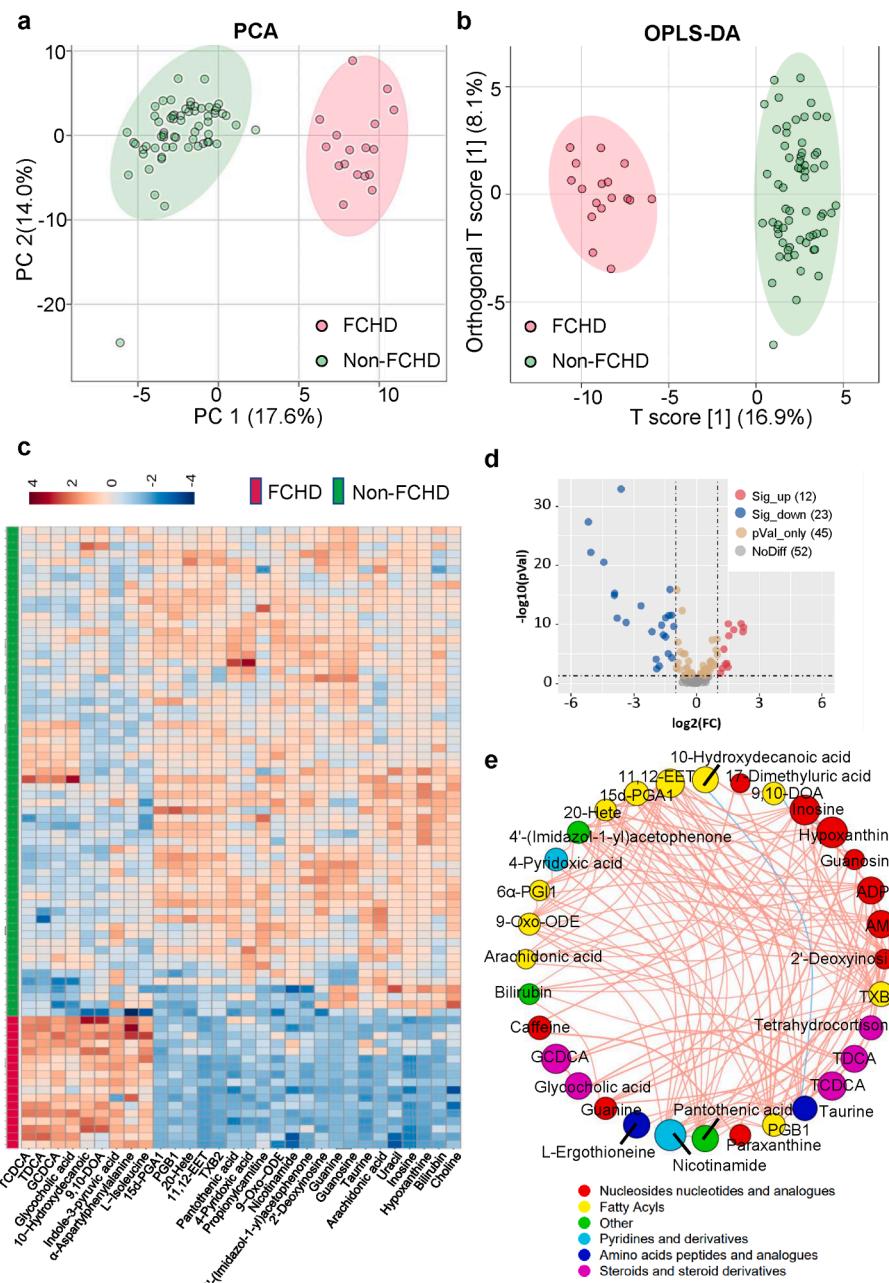


Fig. 1. Metabolomic Profiling of FCHD Maternal Serum Samples. a) PCA score plot discriminating FCHD subjects and non-FCHD subjects. b) OPLS-DA score plot discriminating FCHD subjects and non-FCHD subjects. c) Heatmap of top 30 differential metabolites between the FCHD and the non-FCHD. The values of differential metabolites were normalized and shown as a color scale. d) Volcano plot of differential metabolites between the FCHD and the non-FCHD. e) Correlation network of differential metabolites. Each node represents a compound, and each edge represents the strength of partial correlation between two compounds after conditioning on all other compounds in the datasets.

(%) (False Discovery Rate [FDR] < 0.05). The top 30 differential metabolites were demonstrated by the heatmap (Fig. 1c). The volcano plot highlighted 35 potential metabolic biomarkers, of which $\log_2|\text{FC}| > 1$ and $\text{FDR} < 0.05$ (Fig. 1d) and detailed in Table S2.

To detect the functional groups of metabolites with changes in FCHD during pregnancy, we analyzed the correlations of the 35 compounds (Fig. S3b). Interestingly, network analysis suggested that nucleotides (analogs) and fatty acyl associations with other metabolites are the two most abundant categories. (Fig. 1e).

3.3. Metabolic pathway analysis

Further, we performed metabolic pathway analysis to understand serum metabolic changes. Purine, caffeine, primary bile acid, and arachidonic acid metabolism were significantly enriched (Fig. 2a). There were positive correlations between the seven different metabolites in the purine pathway (Fig. 2b). The strongest correlation was

between inosine and hypoxanthine ($R^2 = 0.94$). The metabolites involved in purine metabolism were mainly nucleotides (analogs), indicating that purine metabolism might be closely related to FCHD. Based on the relationship between compounds in the KEGG database, we visualized the relationship between the seven metabolites (Fig. 2c).

3.4. Establishment of a FCHD diagnostic model based on metabolites

We included 35 different metabolites as candidate variables in the lasso regression for metabolic feature selection (Fig. 3a and b). Ten metabolites, including 4-pyridoxic acid, glycochenodeoxycholic acid, hypoxanthine, (+/-)-9,10-dihydroxy-12Z-octadecenoic acid (9,10-DOA), 10-hydroxydecanoic acid, 11,12-epoxy-(5Z,8Z,11Z)-icosatrienoic acid (11,12-EET), bilirubin, guanine, indole-3-pyruvic acid, and inosine, were then fitted with logistic regression (Called ten-metabolite model). And we further constructed a Nomogram to visualize this ten-metabolite model (Fig. S2c). Unexpectedly, the ROC curve showed that the AUC of

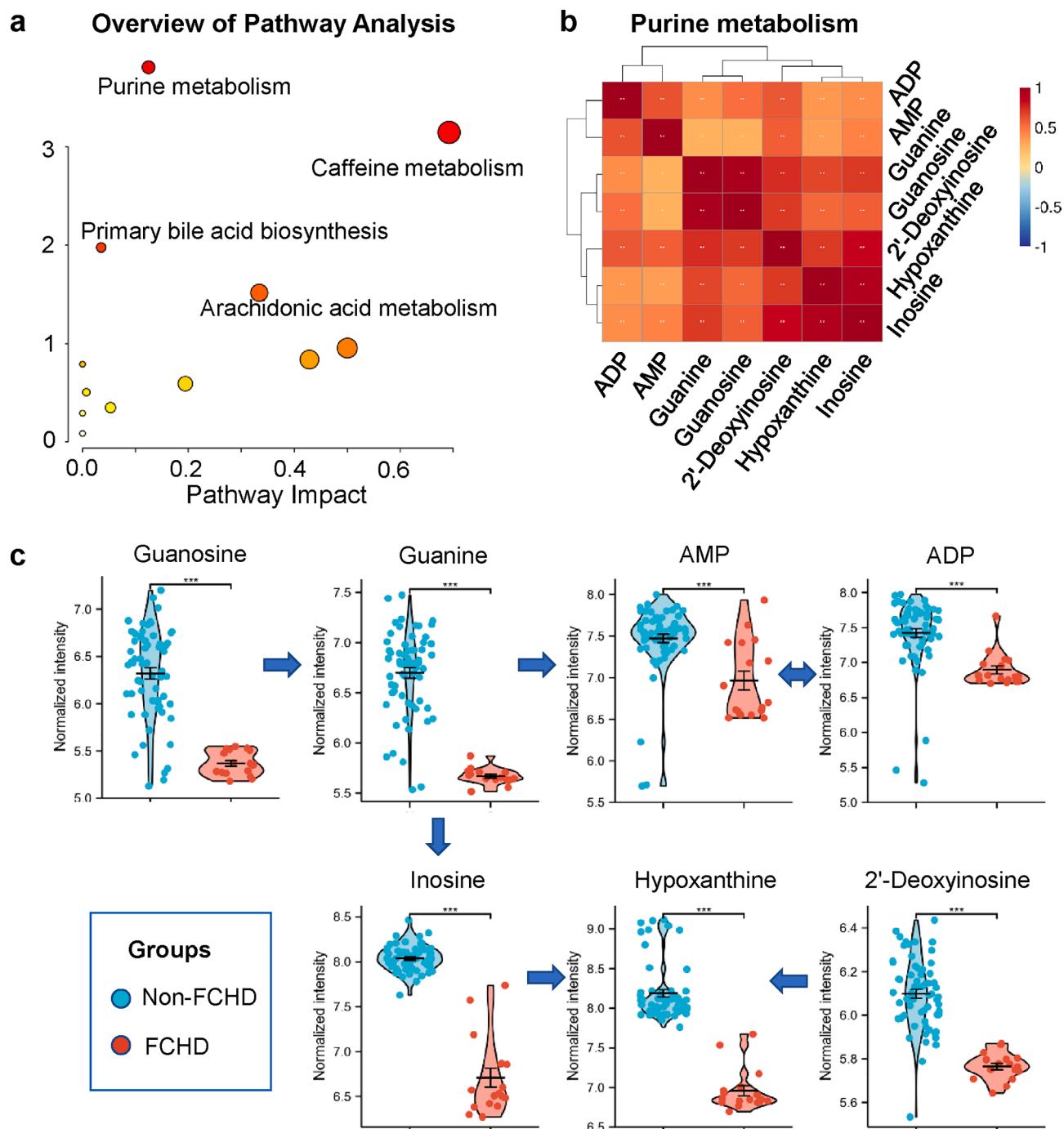


Fig. 2. Enriched KEGG pathways analysis. a) Metabolic pathways undergoing significant changes the FCHD and the non-FCHD group. Red dots (marked) denote pathways with $P < 0.05$. b) Correlation matrix colored by the Pearson correlation coefficient of 7 compounds of Purine metabolism. * $P < 0.05$, ** $P < 0.01$. c) Visualization of the interaction of 7 compounds in purine metabolic pathway based on KEGG database and the relative concentrations of the FCHD and the non-FCHD group. Guanine, guanosine, adenosine 5'-monophosphate (AMP), adenosine diphosphate (ADP), inosine, hypoxanthine, 2'-deoxyinosine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the Nomogram was 1.0 (Fig. 3c). Bootstrap calibration curves, as shown in Fig. 3d, indicate that the prediction of FCHD from the bar chart is consistent with reality (Brier Score < 0.001).

To simplify the ten-metabolite model, we further screened 9,10-DOA and 11,12-EET by stepwise logistic regression analysis, which were incorporated into the final model (Called the two-metabolite model). Fig. 4a shows that the levels of the two metabolites were reversed in CHD and non-CHD groups. The ROC curve showed that the AUC of the two-metabolite model diagnostic model was 1.0 (Fig. 4b). There was no significant difference between the two-metabolite model and the ten-

metabolite model ($P = 0.998$), but the former Akaike Information Criterion (AIC) ($AIC = 6$) was lower than the latter ($AIC = 22$) (Table 2). Similarly, internal validation by bootstrap showed that the calibration curve of the two-metabolites model was consistent with reality (Brier Score = 0.005), and the AUC was still 1.0 (Fig. 4c). In addition, the AUC of 9,10-DOA and 11,12-EET were 0.888 (0.778–0.975) and 0.995 (0.985–1.000), respectively.

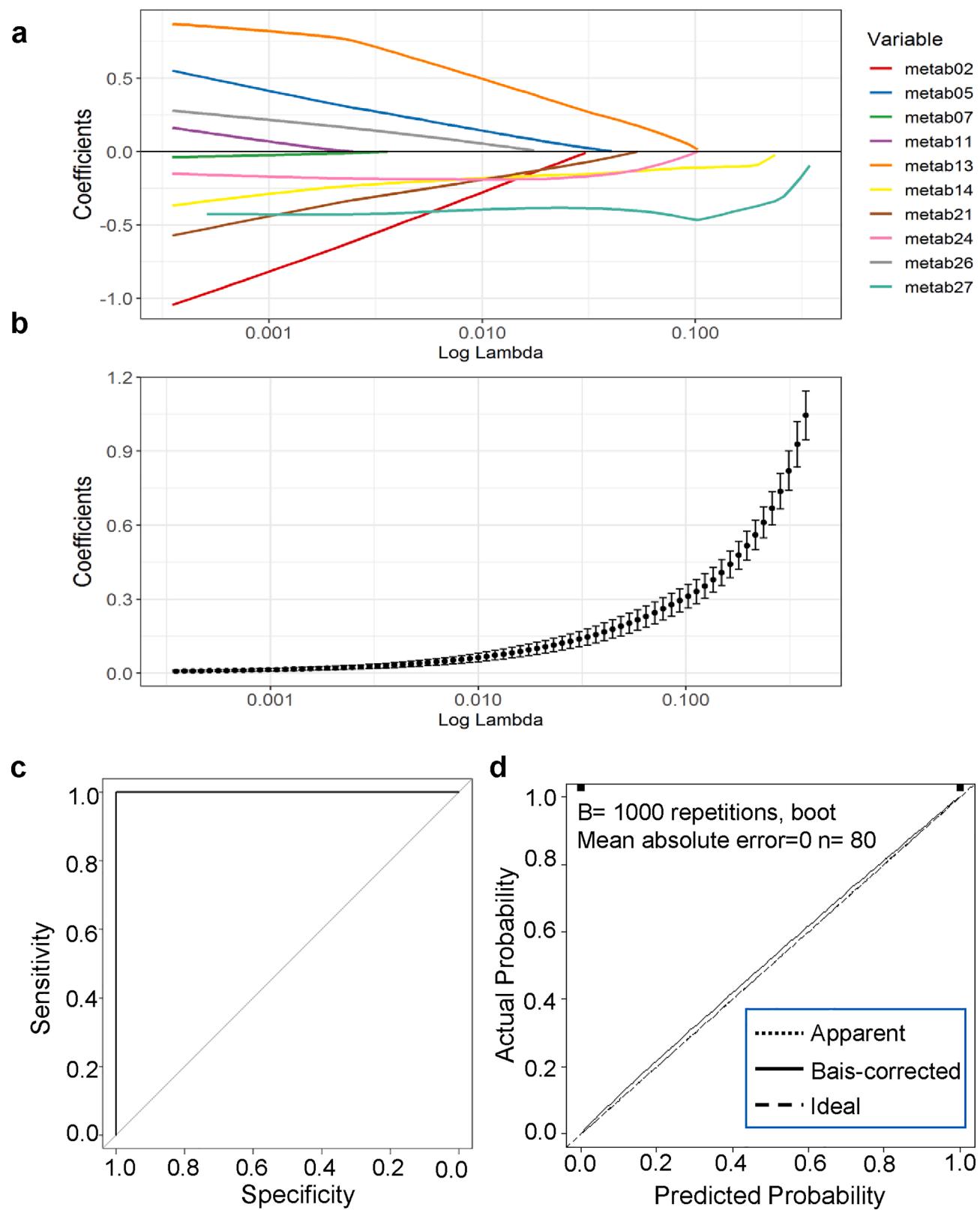


Fig. 3. FCHD model composed with 10 metabolites. a-b) Lasso regression for metabolic feature selection. c) AUC analysis for the ten-metabolite model. d) Bootstrap calibration curves of ten-metabolite model. metab02, 4-pyridoxic acid; metab05, glycochenodeoxycholic acid; metab07, hypoxanthine; metab11, 9,10-DOA; metab13, 10-hydroxydecanoic acid; metab14, 11,12-EET; metab21, bilirubin; metab24, guanine; metab26, indole-3-pyruvic acid; metab27, inosine.

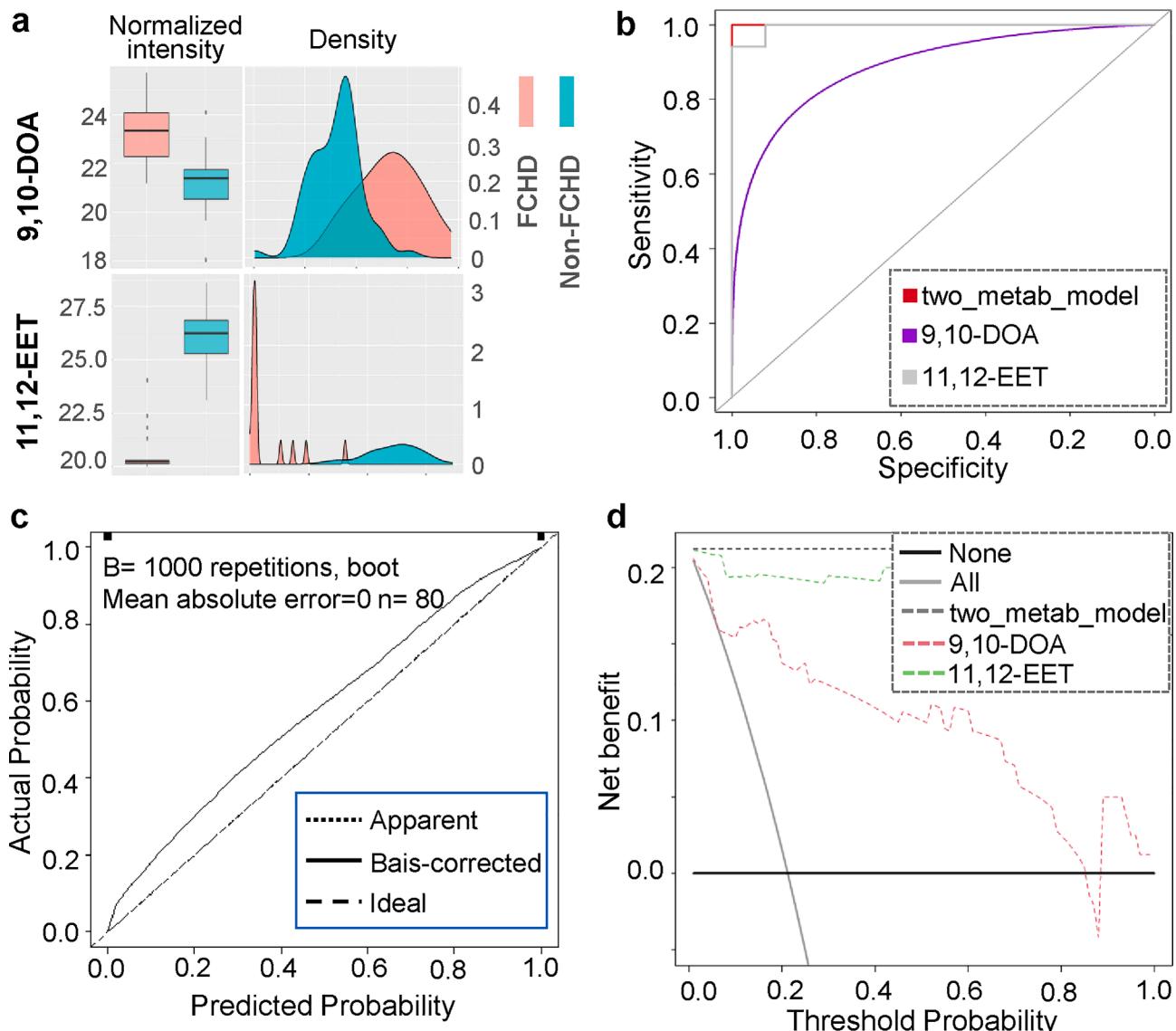


Fig. 4. FCHD model composed with 9,10-DOA and 11,12-EET. a) The levels and distribution of 9,10-DOA and 11,12-EET in CHD and non-CHD group. b) AUC analysis for the two-metabolite model. c) Bootstrap calibration curves of two-metabolite model. d) DCA for two-metabolite model to predict CHD subjects.

Table 2
AUCs of models and metabolites and 95% confidence interval (CI).

Metabolite	AUC	95 % CI	P-value	AIC
Ten-metabolite model	1	/	0.998	22.0
Two-metabolite model	1	/	reference	6.0
9,10-DOA	0.888	0.778–0.975	< 0.001	50.5
11,12-EET	0.995	0.985–1.000	0.003	12.8
Proline	0.867	0.737–0.971	< 0.001	69.4
Uric acid	0.789	0.674–0.887	< 0.001	77.0
Glutamine	0.705	0.556–0.824	< 0.001	78.5
Taurine	0.923	0.784–0.971	< 0.001	35.7

9,10-DOA, (+/-)-9,10-dihydroxy-12Z-octadecenoic acid;
11,12-EET, 11,12-Epoxy-(5Z,8Z,11Z)-icosatrienoic acid.
AUC, Area under the curve.

AIC, Akaike information criterion.

3.5. Comparison of the two-metabolite model with the reported CHD metabolic markers

Previous studies have reported markers in different periods of CHD or different body fluids, involving proline, uric acid, glutamine, and

taurine [15,20]. The distributions of these four metabolites in our study are shown in Fig. S3a. Among the four markers, taurine showed the best discrimination (AUC = 0.923, 95 % CI: 0.784–0.971) but was lower than the two-metabolite model in this study ($P < 0.001$) (Fig. S3b, Table 2). Similarly, on the DCA curve, the clinical benefit of the two-metabolite model was superior to those of the four metabolites (Fig. S3c).

4. Discussion

In this study, we found that the maternal peripheral blood metabolic status of FCHD has changed dramatically. We have also developed a metabolite-based model to assist in the prenatal diagnosis of fetal cardiac malformations. The final simplified model includes two lipid metabolites, 9,10-DOA and 11,12-EET. Although the complexity of the model was reduced, its discrimination was not reduced.

One of the objectives of this study was to understand the serum metabolic profiles of FCHD mothers. Compared with the non-FCHD, 35 metabolite levels in serum were doubling or halving. In this study, PCA and OPLS-DA results showed a perfect clustering between the two groups, indicating that both efficient separation and stable performance were achieved through our analysis. Together, these results indicate that

the maternal metabolism of FCHD changed dramatically. Metabolomics studies in other populations, even in different periods of pregnancy and even in different samples, are consistent with our findings [15,19].

To understand the potential pathogenesis of FCHD, we performed a KEGG enrichment analysis. These differential metabolites were enriched significantly in the purine metabolic pathway, consistent with a previous study [14]. Hypoxanthine, an important purine metabolite, is also characterized by a lower level in adult CHD [21]. Purine metabolism-related nucleotide biosynthesis is associated with the maturation of cardiomyocytes. Specifically, nucleotide starvation directly inhibits the proliferation and maturation of cardiomyocytes, and nucleotide supplementation reverses this effect [22]. Folic acid is an essential cofactor mediating the transfer of one-carbon unit, an important raw material for the synthesis of purines. Sahin-Uysal *et al.* [23] found that the concentrations of folic acid in the umbilical cord of neonatal CHDs were lower than that in healthy controls.

Although the pathogenesis of CHD has been extensively studied and great progress has been made in postpartum treatment, the accurate diagnosis of prenatal CHD is still difficult to obtain satisfactory results. The development of new noninvasive markers may contribute to accurately diagnosing CHD. The initial ten-metabolites model showed excellent discrimination but was very complicated. Therefore, we further screened the metabolites and simplified the model to the two-metabolite model. While improving the utility of the model, it still maintains excellent distinction and is better than 9,10-DOA or 11,12-EET alone. In addition, the internal verification of the bootstrap method has proved that the two-metabolic model is stable. DCA also showed that our secondary metabolite model had a good clinical net benefit, higher than 9,10-DOA or 11,12-EET. The 9,10-DOA in the model, also known as Leukotoxin diol and 9, 10 -DiHOME, is a linoleic acid-derived lipid medium previously reported as a promoter of oxidative stress and inflammation [24]. 11,12-EET is a kind of arachidonic acid metabolite, which affects inflammatory suppression [25]. Although we found that the two lipid mediators associated with proinflammatory/anti-inflammatory activity showed opposite trends in FCHD maternal serum, it is difficult to explain the increase of 9,10-DOA and the decrease of 11,12-EET in FCHD maternal serum based on the present results.

Based on previous studies, uric acid and proline were potential markers of FCHD in the second trimester [15]. Glutamine and taurine have been reported to be potential markers of pediatric CHD in peripheral serum [20]. Our study also validated these four CHD markers, with taurine showing the highest degree of discrimination and net clinical benefit. However, our two-metabolite model performed better. In addition to these four metabolites, other metabolites identified in previous similar studies were not detected or not significantly different in the present study. This may be attributed to the different study populations, sample types and collection times, metabolite extraction methods, equipment, etc.

Our study has the following advantages. Based on the metabolomics of Chinese pregnant women, we first established a potential diagnostic model of FCHD. The model only needs to detect the concentration of two metabolites in maternal peripheral blood to assist in diagnosing FCHD. Moreover, our model performed better than reported metabolic markers. In addition, the peripheral serum is easy to obtain and less invasive, so it can be used as a clinical sample for extensive screening of CHD.

This is a preliminary study and some issues need to be discussed and thoroughly resolved before they can be used clinically. First of all, the sample size of this study is limited. Although the two-metabolite model in our study is excellent, we are cautious about this result. Because this model has not undergone external verification, the stability and applicable scenario need to be further tested.

In summary, the metabolomics study found that FCHD maternal metabolism changes significantly. Most importantly, we screened two key metabolites 9,10-DOA and 11,12-EET, and established an accurate and practical FCHD recognition model. Our findings are of great clinical

significance for the prenatal diagnosis of CHD. The identification model has the potential to serve as a complementary screening procedure for the early detection of FCHD.

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Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study has been reviewed and approved by the Medical Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (2019S065).

CRediT authorship contribution statement

Yiwei Fang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Zheng Zhang:** Data curation, Funding acquisition, Methodology, Validation, Writing - review & editing. **Yun Zhao:** Conceptualization, Writing – review & editing. **Guoqiang Sun:** Writing – review & editing. **Meilin Peng:** Writing – review & editing. **Chunyan Liu:** Writing – review & editing. **Guilin Yi:** Resources, Software, Supervision. **Kai Zhao:** Conceptualization, Project administration, Resources, Supervision, Writing - review & editing. **Hui Yang:** Conceptualization, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Fig. S1. Metabolic profile analysis and FCHD screening model. **Fig. S2.** **a)** Scree plot of PCA model. **b)** Correlation matrix colored by the Pearson correlation coefficient of 35 compounds of significant changed. **c)** Nomogram of the ten-metabolite model for to predict CHD subjects. **Fig. S3.** **a)** The levels and distribution of four metabolites including proline, uric acid, glutamine, and taurine reported previously as a mark of CHD. **b)** Comparison of the two-metabolite model with four known markers in AUC analysis. **c)** Comparison of the two-metabolite model

with four known markers in DCA. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2023.117330>.

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