OBSTETRICS

Metabolomic prediction of fetal congenital heart defect in the first trimester

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OBJECTIVE: The objective of the study was to identify metabolomic markers in maternal first-trimester serum for the detection of fetal congenital heart defects (CHDs).

STUDY DESIGN: Mass spectrometry (direct injection/liquid chromatography and tandem mass spectrometry) and nuclear magnetic resonance spectrometry—based metabolomic analyses were performed between 11 weeks' and 13 weeks 6 days' gestation on maternal serum. A total of 27 CHD cases and 59 controls were compared. There were no known or suspected chromosomal or syndromic abnormalities indicated.

RESULTS: A total of 174 metabolites were identified and quantified using the 2 analytical methods. There were 14 overlapping metabolites between platforms. We identified 123 metabolites that demonstrated significant differences on a univariate analysis in maternal first-trimester serum in CHD vs normal cases. There was a significant disturbance in acylcarnitine, sphingomyelin, and other metabolite levels in CHD pregnancies. Predictive algorithms were developed for CHD detection. High sensitivity (0.929; 95% confidence interval [CI], 0.92—1.00) and specificity (0.932; 95% CI, 0.78—1.00) for CHD detection were achieved (area under the curve, 0.992; 95% CI, 0.973—1.0).

CONCLUSION: In the first such report, we demonstrated the feasibility of the use of metabolomic developing biomarkers for the first-trimester prediction of CHD. Abnormal lipid metabolism appeared to be a significant feature of CHD pregnancies.

Key words: congenital heart defects, maternal first-trimester serum, metabolomic markers

Cite this article as: Bahado-Singh RO, Ertl R, Mandal R, et al. Metabolomic prediction of fetal congenital heart defect in the first trimester. Am J Obstet Gynecol 2014;210:xx-xx.

Congenital heart defect (CHD) is the most important category of congenital anomalies based both on its frequency, 0.6-0.8% of all the births,[1] and health care costs.[2] In contrast to the routine population pregnancy screening for the detection of less common fetal anomalies such as aneuploidies[3] and neural tube defects,[4] there is no comparable screening policy for CHD.

Ultrasound remains the most widely used prenatal tool for the detection of fetal CHD. Although specialist centers that care for high-risk patients report high sensitivities for CHD detection,[5] the overall performance of prenatal ultrasound in the general population remains substantially below[6] that required for an effective screening test. A recent study in the United States found that slightly less than 40% of CHD cases were detected prenatally in a state-wide obstetric population that had an ultrasound examination at the appropriate gestational age.[7]

The overall accuracy of prenatal ultrasound is significantly constrained by its dependence on operator expertise, equipment quality, and uncontrollable variables such as fetal position and maternal obesity. The difficulties associated with the of diagnosis of CHD, moreover, is not limited to the prenatal period because a relatively high percentage of critical CHD fails to be diagnosed in newborns prior to discharge home.[8,9]

The prenatal diagnosis of CHD has distinct advantages including the opportunity for early counseling of families, facilitating reproductive choices, and permitting the transfer of care to expert physicians in tertiary level facilities.[8] Finally, in some categories of CHD prenatal diagnosis reportedly may improve overall outcome compared with those in which the diagnosis is made after birth.[9] An area of concern with respect to the prenatal diagnosis of any congenital anomalies related to potential medical selection against affected fetuses. Data from France have, however, shown that pregnancy termination rates

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Received Dec. 2, 2013; revised Feb. 21, 2014; accepted March 31, 2014.

The study was supported by a grant from the Fetal Medicine Foundation (UK Charity no. 1037116).

The authors report no conflict of interest.

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0002-9378/$36.00 • http://dx.doi.org/10.1016/j.ajog.2014.03.056

have not increased in proportion to improving prenatal diagnosis of CHD.[10] Indeed, termination rates have leveled off and pregnancy termination was exceptional among the more common categories of CHD, whereas at the same time, there has been a reduction in early neonatal deaths.

Metabolomics is a branch of the omics sciences in which high through-put techniques are used for the identification and quantification of the small molecules that constitute the metabolome.[11] Metabolites are a very diverse group of molecules including but not limited to amino acids, nucleic acids, lipids, peptides, sugars, and organic acids. They represent the substrates and byproducts of the various enzymatic reactions within the cells but also respond to and reflect various physiological (eg, age and gender); moreover, pathological and environmental influences including diet, toxins, pharmacological agents and stress, which are important causes and modifiers of disease, significantly influence the metabolome. Based on the latter, metabolomics reportedly may give a more complete description of cellular phenotype than the genome, transcriptome, or proteome.[12]

There has been a dramatic rise in the number of scientific publications related to metabolomics. Increasingly, metabolomics is being used to develop biomarkers for the detection, screening, and monitoring of complex diseases.[13] There is limited prior evidence that CHD may either be caused by or associated with metabolic disturbance in humans.[14,15] Abnormalities of folate and single carbon metabolism has been linked to the development of CHD.[14] To our knowledge, comprehensive metabolomic analysis for the prediction of fetal CHD has not been previously reported.

The objectives of the current study are 2-fold. First, we were interested in determining whether there are significant differences in the first-trimester maternal metabolomic profile in pregnancies with a chromosomally normal fetus compared with those affected with a CHD. Second, we wanted to evaluate metabolite biomarker algorithms that might be useful for the first-trimester prediction of fetal CHD.

Materials and Methods

This study is part of an ongoing prospective study for the first-trimester detection and prediction of fetal and maternal disorders. The details on specimen collection have been extensively described elsewhere.[16,17] The patients were prospectively recruited from an average risk population in Britain between 2006 and 2009. Institutional review board approval was obtained through the Institutional Review Board of King's College Hospital, London, England. Each recruited patient signed a written consent.

Crown rump length (CRL) was used to estimate gestational age. Routine first-trimester screening for aneuploidy is the current standard of care. Maternal demographic and clinical data were obtained along with serum for pregnancy-associated plasma protein-A and free B-human chorionic gonadotropin. Nuchal translucency (NT) thickness was measured for aneuploidy risk estimation. Karyotype and/or newborn examinations were performed to assess chromosomal status. CHD status was determined by prenatal imaging and/or postnatal imaging and based on physical examination in the normal cases.

Samples are immediately transferred to the laboratory within 5 minutes of collection. They are processed after a standing time of 10-15 minutes at room temperature to allow for clotting. The tubes are centrifuged at 3000 rpm for 10 minutes to separate the serum. Then the serum is aliquoted in 8 0.5 mL prelabeled screw tubes (serum) using Gilson micropipette (mark 050). The samples are then subsequently stored in a blue box, previously numbered. The blue box is temporarily stored in a −20°C freezer and then transferred to racks and stored in a −80°C freezer within 24 hours.

We searched our database to identify singleton pregnancies in which the fetus was diagnosed antenatally to have an isolated major cardiac defect with available sample stored at 11−13 weeks' gestation. Cardiac defects were considered to be major if they were lethal or required surgery or interventional cardiac catheterization within the first year of life. We excluded all cases with aneuploidy or noncardiac defects diagnosed prenatally or in the neonatal period. All the CHD diagnoses were made by a specialist in fetal echocardiography. Pregnancies that resulted in live births had newborn confirmation. For cases that underwent termination of pregnancy but for which an autopsy was not performed, the diagnosis was made based on the prenatal examination performed by expert fetal echocardiologists. The study population included 30 cases with major cardiac defects, and each case was matched with 2 controls with no pregnancy complications that were scanned on the same day and that resulted in the live birth of phenotypically normal neonates. A total of 86 sample specimens were processed at the testing laboratory.

Nuclear magnetic resonance metabolomic analysis

In prior publications, we have extensively described the use of the nuclear magnetic resonance (NMR) platform for metabolomic analysis of the serum.[16,17] Serum samples were filtered through 3 kDa cutoff centrifuge filter units (Amicon Micoron YM-3; Sigma-Aldrich, St. Louis, MO) to remove blood proteins. Three hundred fifty microliters of samples was added to the centrifuge filter device and spun (10,000 rpm for 20 minutes) to remove macromolecules such as protein and lipoproteins. If the total volume of sample was less than 300 μL, a 50 mmol NaH_2PO_4 buffer (pH 7) was added to reach a total sample volume of 300 μL. Metabolite concentrations were adjusted for the dilution because of the buffer. Thereafter, 35 μL of D_2O and 15 μL of buffer solution containing (233 Na_2PO_4 at pH 7, 11.667 mmol disodium-2, 2-dimethyl-2-silceptentane-5-sulphonate, and 0.1% NaN_3 in H_2O) were added to the sample.

A total of 350 μL of sample was transferred to a microcell NMR tube (Shigemi, Inc, Allison Park, PA). ^{1}H-NMR spectra were collected on a 500

MHz Inova spectrometer (Varian Inc, Palo Alto, CA) with a 5 mm hydrogen, carbon, and nitrogen Z-gradient pulse field gradient probe. The singlet produced by the disodium-2, 2-dimethyl-2-silceptentane-5-sulphonate methyl groups was used as an internal standard for both chemical shift referencing and for metabolite quantification. The ^{1}H-NMR spectra were analyzed with a Chenomx NMR Suite Professional Software package (version 7.6; Chenomx Inc, Edmonton, ALB, Canada), which permitted both quantitative and qualitative analysis by manually fitting the NMR spectra to an internal metabolite database. Each spectrum was evaluated independently by at least 2 NMR spectroscopists to minimize errors of quantification and identification.

Combined direct injection and liquid chromatography and tandem mass spectrometry compound identification and quantification

We have applied a targeted quantitative metabolomics approach to analyze the serum samples using a combination of direct injection mass spectrometry (Absolute*IDQ* kit) with a reverse-phase liquid chromatography and tandem mass spectrometry (LC-MS/MS) kit. The kit is a commercially available assay from Biocrates Life Sciences AG (Innsbruck, Austria). This kit, in combination with an ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex, Framingham, MA) mass spectrometer, can be used for the targeted identification and quantification of up to 180 different endogenous metabolites including amino acids, acylcarnitines, biogenic amines, glycerophospholipids, sphingolipids, and sugars. The method used combines the derivatization and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring pairs. Isotope-labeled internal standards and other internal standards are integrated into a kit plate filter for metabolite quantification.

The Absolute*IDQ* kit contains a 96 deep-well plate with a filter plate attached with sealing tape and reagents and solvents used to prepare the plate assay. First, 14 wells in the kit were used for 1 blank, 3 zero samples, 7 standards, and 3 quality control samples provided with each kit. All the serum samples were analyzed with the Absolute*IDQ* kit using the protocol described in the Absolute*IDQ* user manual. Briefly, serum samples were thawed on ice and were vortexed and centrifuged at 13,000 × *g*. Ten microliters of each serum sample were loaded onto the center of the filter on the upper 96 well kit plate and dried in a stream of nitrogen. Subsequently, 20 μL of a 5% solution of phenylisothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator.

Extraction of the metabolites was then achieved by adding 300 μL methanol containing 5 mM ammonium acetate. The extracts were obtained by centrifugation into the lower 96 deep-well plate, followed by a dilution step with kit MS running solvent. Mass spectrometric analysis was performed on an API4000 Qtrap tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with a solvent delivery system. The samples were delivered to the mass spectrometer by a liquid chromatography method followed by a DI method. The Biocrates MetIQ software was used to control the entire assay workflow, from sample registration to automated calculation of metabolite concentrations to the

TABLE 1
List of CHD cases

Heart defect	n
AVSD/DORV	1
AVSD/DORV/PA	1
DORV/PS	2
DORV/TOF	2
DORV/PA	1
TGA	3
TGA-corrected VSD	1
TGA/PS	1
TOF	9
TOF/MS	1
TOF/PA	5

AVSD, atrioventricular septal defect; *CHD*, congenital heart defect; *DORV*, double outlet right ventricle; *MS*, mitral stenosis; *PA*, pulmonary atresia; *PS*, pulmonary valve stenosis; *TGA*, transposition of the great artery; *TOF*, tetralogy of Fallot; *VSD*, ventricular septal defect.

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TABLE 2
Maternal demographic and medical characteristics: comparison of CHD and control groups

Parameter	CHD	Control	*P* value
n	27	59	
Mean maternal age (y), mean (SD)[a]	29.2 (6.5)	30.0 (5.2)	NS
Ethnicity, n (%)[b]			
Caucasian	23 (82.1)	47 (79.7)	NS
African descent	3 (10.7)	10 (16.9)	
Asian/other	2 (7.1)	2 (3.4)	
Nulliparous, n (%)[c]			NS
Multiparous	12 (42.9)	23 (39.0)	
Nulliparous	16 (57.1)	36 (61.0)	
BMI, mean (SD)[a]	24.1 (4.2)	24.4 (3.5)	NS
GA-CRL (wks), mean (SD)[a]	12.7 (0.7)	12.7 (0.6)	NS

BMI, body mass index; *CHD*, congenital heart defect; *GA-CRL*, gestational age in weeks based on crown rump length; *NS*, not significant.

[a] Independent sample t test; [b] χ^2 test; [c] Fisher exact test.

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TABLE 3
Univariate analysis for DI/LC-MS/MS: CHD vs control

Metabolite (biochemical name)	Mean (SD) CHD	Mean (SD) Control	Fold change	CHD/control	*P* value[a]
Number of cases	27	59	—	—	—
C0 (carnitine)	16.798 (14.323)	24.408 (5.444)	−1.45	Down	.0168
C2 (acetylcarnitine)	2.5022 (2.2864)	4.4217 (1.7936)	−1.77	Down	.0008
C3 (propionylcarnitine)	0.2075 (0.1802)	0.2999 (0.081)	−1.45	Down	.0177
C3:1 (propenoylcarnitine)	0.0253 (0.00163)	0.0317 (0.0061)	−1.26	Down	.0363
C3-OH (hydroxypropionylcarnitine)	0.0918 (0.0644)	0.1834 (0.0256)	−2	Down	.0000
C4 (butyrylcarnitine)	0.1378 (0.119)	0.2169 (0.0736)	−1.57	Down	.0025
C5 (valerylcarnitine)	0.0845 (0.0722)	0.1179 (0.0241)	−1.39	Down	.0070
C5-M-DC (methylglutarylcarnitine)	0.049 (0.0327)	0.1123 (0.0447)	−2.29	Down	.0000
C5-OH(C3-DC-M) (hydroxyvalerylcarnitine (methylmalonylcarnitine))	0.0602 (0.0527)	0.1732 (0.0719)	−2.88	Down	.0000
C5:1-DC (glutaconylcarnitine)	0.0725 (0.1405)	0.0262 (0.005)	2.76	Up	.0130
C6:1 (hexenoylcarnitine)	0.0241 (0.0125)	0.0293 (0.0049)	−1.22	Down	.0050
C8 (octanoylcarnitine)	0.0943 (0.04369)	0.1304 (0.0496)	−1.38	Down	.0018
C9 (nonaylcarnitine)	0.0353 (0.0207)	0.059 (0.017)	−1.67	Down	.0000
C10 (decanoylcarnitine)	0.1267 (0.0743)	0.2449 (0.1076)	−1.93	Down	.0000
C10:1 (decenoylcarnitine)	0.204 (0.0677)	0.2306 (0.0504)	−1.13	Down	.0045
C10:2 (decadienylcarnitine)	0.0317 (0.0179)	0.0498 (0.0156)	−1.57	Down	.0001
C12 (dodecanoylcarnitine)	0.045 (0.0197)	0.0651 (0.0228)	−1.45	Down	.0003
C14 (tetradecanoylcarnitine)	0.0337 (0.0126)	0.0403 (0.0065)	−1.19	Down	.0003
C14:1 (tetradecenoylcarnitine)	0.1014 (0.0757)	0.1978 (0.0404)	−1.95	Down	.0000
C14:2 (tetradecadienylcarnitine)	0.0135 (0.0071)	0.0217 (0.0087)	−1.6	Down	.0001
C14:2-OH (hydroxytetradecadienylcarnitine)	0.0105 (0.0063)	0.0125 (0.0042)	−1.2	Down	.0039
C16 (hexadecanoylcarnitine)	0.046 (0.0375)	0.0767 (0.0217)	−1.67	Down	.0000
C16:2 (hexadecadienylcarnitine)	0.011 (0.0057)	0.0133 (0.0024)	−1.21	Down	.0068
C18 (octadecanoylcarnitine)	0.0258 (0.018)	0.0371 (0.0085)	−1.44	Down	.0005
C18:1 (octadecenoylcarnitine)	0.0477 (0.043)	0.0835 (0.037)	−1.75	Down	.0000
C18:2 (octadecadienylcarnitine)	0.0208 (0.0151)	0.0321 (0.0112)	−1.54	Down	.0011
LysoPC a C16:0 (lysophosphatidylcholine acyl C16:0)	72.189 (66.133)	142.065 (39.638)	−1.97	Down	.0000
LysoPC a C16:1 (lysophosphatidylcholine acyl C16:1)	1.8565 (1.8622)	2.6836 (1.0076)	−1.45	Down	.0155
LysoPC a C17:0 (lysophosphatidylcholine acyl C17:0)	1.8811 (1.9996)	2.6388 (0.8126)	−1.4	Down	.0044
LysoPC a C18:0 (lysophosphatidylcholine acyl C18:0)	19.323 (17.977)	36.487 (11.673)	−1.89	Down	.0001
LysoPC a C18:1 (lysophosphatidylcholine acyl C18:1)	13.380 (12.740)	27.946 (9.468)	−2.09	Down	.0000
LysoPC a C18:2 (lysophosphatidylcholine acyl C18:2)	17.582 (17.132)	36.345 (14.315)	−2.07	Down	.0000
LysoPC a C20:3 (lysophosphatidylcholine acyl C20:3)	1.6375 (1.5461)	2.7411 (1.1319)	−1.67	Down	.0011
LysoPC a C20:4 (lysophosphatidylcholine acyl C20:4)	4.2194 (3.7183)	7.9856 (2.2556)	−1.89	Down	.0000
PC aa C28:1 (phosphatidylcholine diacyl C28:1)	2.3011 (1.9755)	3.4444 (1.1203)	−1.5	Down	.0168
PC aa C30:0 (phosphatidylcholine diacyl C30:0)	3.5667 (3.29)	6.0819 (2.7436)	−1.71	Down	.0015
PC aa C30:2 (phosphatidylcholine diacyl C30:2)	0.2731 (0.3429)	0.5917 (0.1918)	−2.17	Down	.0000

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(continued)

TABLE 3
Univariate analysis for DI/LC-MS/MS: CHD vs control *(continued)*

Metabolite (biochemical name)	Mean (SD) CHD	Mean (SD) Control	Fold change	CHD/control	*P* value[a]
PC aa C32:0 (phosphatidylcholine diacyl C32:0)	11.830 (10.249)	21.468 (6.561)	−1.81	Down	.0003
PC aa C32:1 (phosphatidylcholine diacyl C32:1)	12.399 (12.890)	23.352 (12.85)	−1.88	Down	.0003
PC aa C32:2 (phosphatidylcholine diacyl C32:2)	3.395 (3.2336)	6.5874 (2.6463)	−1.94	Down	.0001
PC aa C32:3 (phosphatidylcholine diacyl C32:3)	0.5258 (0.4654)	0.8819 (0.2227)	−1.68	Down	.0014
PC aa C34:1 (phosphatidylcholine diacyl C34:1)	168.650 (151.577)	316.441 (96.259)	−1.88	Down	.0001
PC aa C34:2 (phosphatidylcholine diacyl C34:2)	284.991 (253.246)	512.414 (109.171)	−1.8	Down	.0002
PC aa C34:3 (phosphatidylcholine diacyl C34:3)	14.688 (13.192)	26.705 (10.794)	−1.82	Down	.0006
PC aa C34:4 (phosphatidylcholine diacyl C34:4)	1.580 (1.4083)	2.9268 (1.229)	−1.85	Down	.0003
PC aa C36:1 (phosphatidylcholine diacyl C36:1)	34.645 (31.864)	65.276 (21.905)	−1.88	Down	.0000
PC aa C36:2 (phosphatidylcholine diacyl C36:2)	155.673 (140.706)	279.018 (74.520)	−1.79	Down	.0001
PC aa C36:3 (phosphatidylcholine diacyl C36:3)	106.650 (95.964)	188.801 (66.714)	−1.77	Down	.0009
PC aa C36:4 (phosphatidylcholine diacyl C36:4)	139.129 (121.517)	260.540 (70.103)	−1.87	Down	.0000
PC aa C36:5 (phosphatidylcholine diacyl C36:5)	20.626 (20.914)	42.042 (23.098)	−2.04	Down	.0001
PC aa C36:6 (phosphatidylcholine diacyl C36:6)	1.2059 (1.1361)	2.1289 (0.9321)	−1.77	Down	.0015
PC aa C38:0 (phosphatidylcholine diacyl C38:0)	3.0315 (2.754)	5.089 (1.5576)	−1.68	Down	.0013
PC aa C38:1 (phosphatidylcholine diacyl C38:1)	1.0299 (1.2723)	1.4582 (0.58)	−1.42	Down	.0026
PC aa C38:3 (phosphatidylcholine diacyl C38:3)	39.351 (35.404)	67.848 (22.399)	−1.72	Down	.0015
PC aa C38:4 (phosphatidylcholine diacyl C38:4)	76.611 (64.780)	141.130 (37.687)	−1.84	Down	.0001
PC aa C38:5 (phosphatidylcholine diacyl C38:5)	40.448 (34.594)	76.975 (21.930)	−1.9	Down	.0001
PC aa C38:6 (phosphatidylcholine diacyl C38:6)	90.567 (84.144)	178.280 (54.427)	−1.97	Down	.0000
PC aa C40:1 (phosphatidylcholine diacyl C40:1)	0.5856 (0.3525)	0.6059 (0.1541)	−1.03	Down	.0255
PC aa C40:4 (phosphatidylcholine diacyl C40:4)	2.9053 (2.5455)	4.9576 (1.7367)	−1.71	Down	.0017
PC aa C40:5 (phosphatidylcholine diacyl C40:5)	7.8919 (6.8919)	14.0773 (4.5583)	−1.78	Down	.0005
PC aa C40:6 (phosphatidylcholine diacyl C40:6)	28.029 (25.525)	54.476 (16.859)	−1.94	Down	.0000
PC aa C42:0 (phosphatidylcholine diacyl C42:0)	0.6763 (0.5907)	1.2374 (0.3778)	−1.83	Down	.0000
PC aa C42:1 (phosphatidylcholine diacyl C42:1)	0.3311 (0.3002)	0.5457 (0.1542)	−1.65	Down	.0009
PC aa C42:2 (phosphatidylcholine diacyl C42:2)	0.2295 (0.2297)	0.3178 (0.1022)	−1.38	Down	.0130
PC aa C42:4 (phosphatidylcholine diacyl C42:4)	0.2195 (0.2094)	0.2922 (0.08)	−1.33	Down	.0200
PC aa C42:5 (phosphatidylcholine diacyl C42:5)	0.5168 (0.4713)	0.8146 (0.2572)	−1.58	Down	.0052
PC aa C42:6 (phosphatidylcholine diacyl C42:6)	0.9336 (0.4475)	1.1482 (0.298)	−1.23	Down	.0123
PC ae C32:1 (phosphatidylcholine acly-alkyl C32:1)	2.2309 (1.9781)	3.8034 (1.1018)	−1.7	Down	.0009
PC ae C32:2 (phosphatidylcholine acly-alkyl C32:2)	0.6953 (0.632)	1.0491 (0.2574)	−1.51	Down	.0085
PC ae C34:0 (phosphatidylcholine acly-alkyl C34:0)	1.237 (1.1026)	1.9551 (0.6817)	−1.58	Down	.0045
PC ae C34:1 (phosphatidylcholine acly-alkyl C34:1)	7.9483 (7.1334)	14.6214 (4.8614)	−1.84	Down	.0002
PC ae C34:2 (phosphatidylcholine acly-alkyl C34:2)	9.0328 (8.2812)	15.6214 (5.1054)	−1.73	Down	.0004
PC ae C34:3 (phosphatidylcholine acly-alkyl C34:3)	6.5576 (6.1098)	11.1413 (3.0949)	−1.7	Down	.0003
PC ae C36:0 (phosphatidylcholine acly-alkyl C36:0)	1.0045 (1.1707)	1.0445 (0.3054)	−1.04	Down	.0195
PC ae C36:1 (phosphatidylcholine acly-alkyl C36:1)	7.0628 (6.4818)	11.3303 (3.5905)	−1.6	Down	.0048

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TABLE 3
Univariate analysis for DI/LC-MS/MS: CHD vs control *(continued)*

Metabolite (biochemical name)	Mean (SD) CHD	Mean (SD) Control	Fold change	CHD/control	*P* value[a]
PC ae C36:2 (phosphatidylcholine acly-alkyl C36:2)	12.106 (10.981)	20.838 (6.545)	−1.72	Down	.0004
PC ae C36:3 (phosphatidylcholine acly-alkyl C36:3)	6.3834 (5.9313)	11.0311 (3.8796)	−1.73	Down	.0005
PC ae C36:4 (phosphatidylcholine acly-alkyl C36:4)	12.696 (10.967)	21.900 (6.566)	−1.72	Down	.0008
PC ae C36:5 (phosphatidylcholine acly-alkyl C36:5)	8.0333 (6.9636)	14.273 (3.6587)	−1.78	Down	.0001
PC ae C38:0 (phosphatidylcholine acly-alkyl C38:0)	2.4102 (2.0677)	3.9207 (1.302)	−1.63	Down	.0034
PC ae C38:1 (phosphatidylcholine acly-alkyl C38:1)	0.8754 (1.2408)	0.8763 (0.4078)	−1.00	Down	.0114
PC ae C38:2 (phosphatidylcholine acly-alkyl C38:2)	1.9462 (1.9687)	3.0116 (0.9756)	−1.55	Down	.0013
PC ae C38:3 (phosphatidylcholine acly-alkyl C38:3)	3.9018 (3.5774)	6.0499 (2.145)	−1.55	Down	.0100
PC ae C38:4 (phosphatidylcholine acly-alkyl C38:4)	9.647 (8.2858)	17.6932 (5.2507)	−1.83	Down	.0001
PC ae C38:5 (phosphatidylcholine acly-alkyl C38:5)	11.949 (10.363)	22.833 (6.419)	−1.91	Down	.0000
PC ae C38:6 (phosphatidylcholine acly-alkyl C38:6)	6.044 (5.4155)	11.0441 (3.144)	−1.83	Down	.0002
PC ae C40:1 (phosphatidylcholine acly-alkyl C40:1)	1.0876 (1.0198)	1.7633 (0.55)	−1.62	Down	.0011
PC ae C40:2 (phosphatidylcholine acly-alkyl C40:2)	1.6831 (1.5317)	2.6479 (0.753)	−1.57	Down	.0062
PC ae C40:3 (phosphatidylcholine acly-alkyl C40:3)	1.2038 (1.1703)	1.6982 (0.5214)	−1.41	Down	.0155
PC ae C40:4 (phosphatidylcholine acly-alkyl C40:4)	1.886 (1.6743)	3.2221 (1.0074)	−1.71	Down	.0017
PC ae C40:5 (phosphatidylcholine acly-alkyl C40:5)	3.1709 (2.7955)	5.6119 (1.6168)	−1.77	Down	.0004
PC ae C40:6 (phosphatidylcholine acly-alkyl C40:6)	4.8457 (4.4043)	9.0374 (2.6279)	−1.87	Down	.0000
PC ae C42:0 (phosphatidylcholine acly-alkyl C42:0)	0.8517 (0.4519)	1.0654 (0.2901)	−1.25	Down	.0048
PC ae C42:1 (phosphatidylcholine acly-alkyl C42:1)	0.3911 (0.3516)	0.5233 (0.162)	−1.34	Down	.0237
PC ae C42:2 (phosphatidylcholine acly-alkyl C42:2)	0.5345 (0.5106)	0.8615 (0.2624)	−1.61	Down	.0015
PC ae C42:3 (phosphatidylcholine acly-alkyl C42:3)	0.8538 (0.817)	1.3914 (0.4665)	−1.63	Down	.0019
PC ae C42:4 (phosphatidylcholine acly-alkyl C42:4)	1.0104 (0.9584)	1.6802 (0.6029)	−1.66	Down	.0027
PC ae C42:5 (phosphatidylcholine acly-alkyl C42:5)	2.2948 (1.8271)	3.9456 (1.2567)	−1.72	Down	.0002
PC ae C44:3 (phosphatidylcholine acly-alkyl C44:3)	0.1417 (0.1267)	0.203 (0.0655)	−1.43	Down	.0021
PC ae C44:4 (phosphatidylcholine acly-alkyl C44:4)	0.4641 (0.421)	0.7322 (0.2906)	−1.58	Down	.0020
PC ae C44:5 (phosphatidylcholine acly-alkyl C44:5)	2.0303 (1.7939)	3.7191 (1.3549)	−1.83	Down	.0001
PC ae C44:6 (phosphatidylcholine acly-alkyl C44:6)	1.3903 (1.2122)	2.5122 (0.742)	−1.81	Down	.0001
SM (OH) C14:1 (hydroxysphingomyeline C14:1)	4.7973 (4.3326)	7.9934 (2.0106)	−1.67	Down	.0009
SM (OH) C16:1 (hydroxysphingomyeline C16:1)	2.6565 (2.2818)	4.4412 (0.9153)	−1.67	Down	.0012
SM (OH) C22:1 (hydroxysphingomyeline C22:1)	9.7724 (8.4566)	16.7107 (4.144)	−1.71	Down	.0021
SM (OH) C22:2 (hydroxysphingomyeline C22:2)	9.1052 (7.7116)	15.5155 (3.1796)	−1.7	Down	.0009
SM (OH) C24:1 (hydroxysphingomyeline C24:1)	1.0876 (0.9485)	1.7359 (0.44)	−1.6	Down	.0092
SM C16:0 (sphingomyeline C16:0)	79.934 (68.457)	140.997 (28.289)	−1.76	Down	.0002
SM C16:1 (sphingomyeline C16:1)	11.455 (10.081)	20.316 (4.306)	−1.77	Down	.0002
SM C18:0 (sphingomyeline C18:0)	18.125 (15.986)	31.102 (6.468)	−1.72	Down	.0003
SM C18:1 (sphingomyeline C18:1)	8.2855 (7.2093)	14.7 (3.1262)	−1.77	Down	.0002
SM C20:2 (sphingomyeline C20:2)	0.8174 (0.8328)	1.7089 (0.4626)	−2.09	Down	.0000
SM C22:3 (sphingomyeline C22:3)	4.5683 (5.4572)	13.3436 (3.8124)	−2.92	Down	.0000

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TABLE 3
Univariate analysis for DI/LC-MS/MS: CHD vs control *(continued)*

Metabolite (biochemical name)	Mean (SD) CHD	Mean (SD) Control	Fold change	CHD/control	*P* value[a]
SM C24:0 (sphingomyeline C24:0)	16.752 (14.327)	29.457 (7.463)	−1.76	Down	.0008
SM C24:1 (sphingomyeline C24:1)	43.053 (37.370)	79.007 (16.719)	−1.84	Down	.0000
SM C26:0 (sphingomyeline C26:0)	0.1904 (0.2071)	0.2502 (0.0652)	−1.31	Down	.0074
SM C26:1 (sphingomyeline C26:1)	0.3279 (0.2907)	0.5388 (0.145)	−1.64	Down	.0057

Concentration values are in μM/L (micromoles per Litre).

CHD, congenital heart defect; *DI/LC-MS/MS*, direct injection liquid chromatography and tandem mass spectrometry.

[a] Wilcoxon-Mann-Whitney test's *P* value.

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export of data into other data analysis programs.

A targeted profiling scheme was used to quantitatively screen for known small molecule metabolites using multiple reaction monitoring, neutral loss, and precursor ion scans. The metabolomic analyses were performed at the Metabolomics Innovation Centre, University of Alberta, Edmonton, Canada.

Statistical analysis

Analysis of the metabolomics data was performed with the MetaboAnalyst web-based statistical package.[18] Univariate analysis of continuous data was conducted using Wilcoxon-Mann-Whitney test, and categorical data were analyzed using Pearson χ^2 and Fisher exact tests. Multivariate analyses were conducted using binary logistic regression with selected features using a Lasso algorithm. A significance level of $P < .05$ was used to define statistical significance.

Three different sets of analyses were performed. Metabolites were analyzed by themselves and also with the addition of demographic characteristics such as ethnicity, body mass index (BMI), parity, and an ultrasound measurement of fetal length (ie, CRL). Finally metabolites with NT thickness were evaluated. It should be pointed out that the CRL is the most precise measure of gestational age and therefore used to assess whether first-trimester gestational age affected the maternal serum level of the metabolites.

Data normalization of metabolite concentration is critical to creating a normal or Gaussian distribution. Normalization allows conventional statistical tests to be performed, and it simplifies data interpretation. In this study, we used log-transformed metabolite values.

Principal component analysis (PCA) is a multivariate analysis technique[18] and was used to find the most useful principal components for distinguishing groups of interest in the dataset. The first principal component has the largest possible variance to discriminate each group, and the second principal component that is calculated orthogonal to the first principal component has the second highest variance possible.

Partial least squares discriminant analysis (PLS-DA) rotates around the different principal components to find the optimal combination for discriminating the case from the control group.[19] Permutation analysis uses random resampling of cases and controls to determine the probability that the observed and control groups is a result of chance. A total of 2000 resamplings were performed and calculated. A *P* value that represents the probability of a chance finding is generated. A variable importance in projection (VIP) plot,[19] which is a visual representation of the significance or importance of the particular metabolite in discriminating the groups of interest, is provided.

Metabolite concentrations in CHD vs controls were compared. Logistic regression analyses were performed with outcomes (CHD or normal) as the dependent variable and metabolites as the independent or determinant variable to develop a predictive algorithm for CHD detection. Metabolites with a significant correlation with CHD status on univariate analysis were initially entered into the model development. Other variables including NT, fetal CRL, and maternal demographics and medical status were combined with metabolite concentrations and run in selected logistic regression analyses. Finally, logistic regression analyses including NT and the preceding metabolomic and maternal markers were performed.

Paired sensitivity and false-positive rates (1 − specificity) at different risk thresholds were calculated. A receiver-operator characteristic (ROC) curve is plotted with sensitivity values on the Y-axis and the corresponding false-positive ratio (1 − specificity) on the X-axis. The area under the ROC curve (AUC) indicates the accuracy of a test for correctly distinguishing one group such as CHD pregnancies from normal (control), where AUC = 1 indicates a perfectly discriminating test. The 95% confidence interval (CI) and *P* values for the AUC curves were calculated. Permutation testing was also performed to determine the probability that the AUC obtained was due to chance.

RESULTS

Metabolomic analyses using 2 analytical techniques, NMR and direct injection (DI)/LC-MS/MS, were performed for 27 cases of CHD and 59 normal matched controls. Neither case nor control fetuses had any known or suspected

TABLE 4
Univariate analysis for NMR: CHD vs control

	Mean (SD)				
Metabolite	CHD	Control	Fold change	CHD/control	*P* value[a]
Number of cases	27	59	—	—	—
2-Hydroxybutyrate	17.13 (7.48)	16.69 (6.25)	1.03	Up	.8963
3-Hydroxybutyrate	33.63 (42.57)	30.15 (37.69)	1.12	Up	.4208
Acetamide	6.56 (3.47)	7.78 (5.04)	−1.19	Down	.5828
Acetate	25.69 (7.99)	29.85 (8.26)	−1.16	Down	.0207[b]
Acetoacetate	14.33 (10.92)	12.64 (8.21)	1.13	Up	.6822
Acetone	15 (4.34)	18.46 (5.83)	−1.23	Down	.0039[b]
Alanine	284.62 (65.96)	267.32 (53.39)	1.06	Up	.1440
Betaine	21.99 (7.84)	20.05 (8.39)	1.1	Up	.2056
Carnitine	21.08 (4.88)	19.98 (4.82)	1.06	Up	.1894
Choline	7.68 (3.02)	7.49 (2.69)	1.03	Up	.8123
Citrate	59.29 (11.58)	61.85 (13.38)	−1.04	Down	.4510
Creatine	25.5 (11.41)	23.84 (11.5)	1.07	Up	.3449
Creatinine	36.96 (8.1)	35.54 (9.46)	1.04	Up	.1879
Dimethyl sulfone	5.06 (3.56)	4.78 (2.73)	1.06	Up	.9703
Ethanol	46.68 (25.73)	32.21 (16.46)	1.45	Up	.013[b]
Glucose	3241.19 (898.95)	3171.93 (754.22)	1.02	Up	.7376
Glutamate	52.21 (14.83)	56.4 (12.69)	−1.08	Down	.1147
Glutamine	311.3 (55.65)	310.98 (52.92)	1	Up	1.0000
Glycerol	124.62 (45.58)	133.9(34.38)	−1.07	Down	.1614
Glycine	141.82 (37.97)	135.06 (40.06)	1.05	Up	.2040
Isobutyrate	4.59 (1.89)	4.65 (2.06)	−1.01	Down	.9888
Isoleucine	43.24 (16.72)	40.9 (11.7)	1.06	Up	.6652
Lactate	1236.89 (410.48)	1270.97 (604.28)	−1.03	Down	.7341
Leucine	73.74 (23.04)	69.55 (16.39)	1.06	Up	.7028
Lysine	103.04 (27.01)	97.44 (29.48)	1.06	Up	.2073
Malonate	11.84 (2.88)	11.11 (3)	1.07	Up	.1754
Methionine	16.34 (4.74)	15.3 (4.66)	1.07	Up	.2317
Ornithine	25.15 (7.57)	22.37 (8.47)	1.12	Up	.1365
Phenylalanine	44.46 (12.81)	43.9 (15.78)	1.01	Up	.5058
Proline	111.43 (37.73)	106 (33.59)	1.05	Up	.5484
Propylene glycol	8.84 (2.78)	8.25 (2.09)	1.07	Up	.4047
Pyruvate	63.92 (19.24)	54.52 (25.5)	1.17	Up	.0155[b]
Serine	75.47 (22.9)	77.55 (23.11)	−1.03	Down	.9370
Succinate	3.35 (1.78)	3.31 (1.13)	1.01	Up	.5506
Threonine	112.57 (25.66)	107.31 (26.21)	1.05	Up	.3449
Tyrosine	42.94 (11.38)	45.63 (19.15)	−1.06	Down	.9296

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(continued)

TABLE 4
Univariate analysis for NMR: CHD vs control *(continued)*

Metabolite	Mean (SD) CHD	Mean (SD) Control	Fold change	CHD/control	*P* value[a]
Valine	147.88 (37.36)	143.24 (30.09)	1.03	Up	.5148
3-Methylhistidine	26.93 (13.26)	23.74 (15.16)	1.13	Up	.0185[b]

CHD, congenital heart defect; *NMR*, nuclear magnetic resonance.

[a] Wilcoxon-Mann-Whitney test's *P* value; [b] statistically significant ($P < .05$).

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chromosomal or syndromic abnormalities. Table 1 gives the breakdown of the different types of CHD. Table 2 compares maternal pregnancy and other demographic characteristics between study and control groups. No significant difference was observed. A total of 150 metabolites were identified and quantified using the DI/LC-MS/MS technique. By using NMR spectroscopy, a total of 38 metabolites were quantified. There were 174 distinct metabolites measured by the 2 platforms.

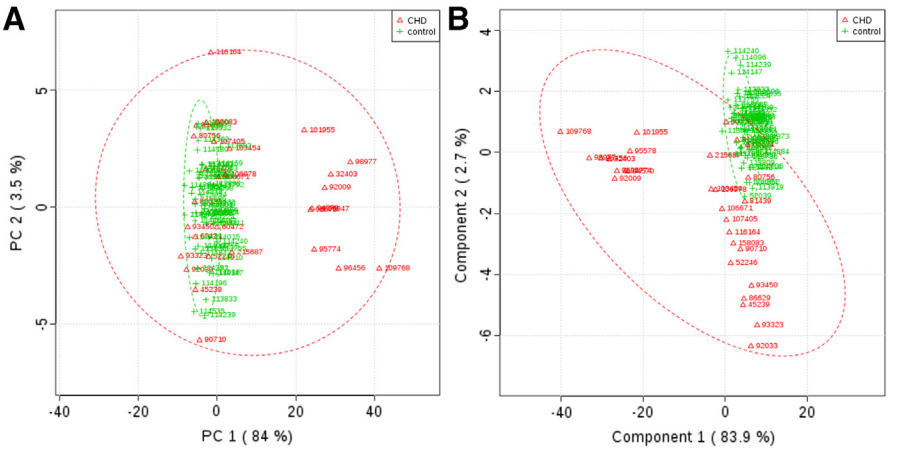
By using a univariate analysis, a total of 118 metabolites from the DI/LC-MS/MS assay were found to have significant concentration differences in maternal serum in CHD vs normal controls on paired comparisons. The mean (SD) concentrations, Wilcoxon-Mann-Whitney test, and *P* values for each of those significant metabolites along with fold change and direction of change in CHD cases relative to controls are provided in Table 3.

In Table 4, a similar comparison of metabolite concentrations was performed for only NMR-based metabolomics. Significant differences were noted in 5 metabolites using the NMR platform.

The separation between the CHD cases and controls from the PCA analysis of the DI/LC-MS/MS data is shown in Figure 1, A. The PLS-DA analysis resulted in a clear separation between the groups (Figure 1, B). Permutation testing demonstrated that the observed separation was not by chance ($P < .0005$). A VIP plot of the PLS-DA in which the metabolites were ranked by their contribution to distinguishing the CHD from control groups is shown in Figure 2. The plot shows the top 15 important metabolites. The greater the distance from the Y-axis, the greater is the contribution of a particular metabolite in distinguishing cases from controls. The heat map on the right side of this plot also indicates whether the particular metabolite's concentration is increased or decreased in CHD relative to controls. The VIP plot indicated that several acylcarnitines such as hydroxypropionylcarnitine (C3-OH), C5-OH(C3-DC-M), C14:1, and sphingomyelin SM C22:3 were the most discriminating metabolites for separating CHD cases from normal control specimens. The heat map on the right of the Y-axis indicates that C3-OH, C5-OH(C3-DC-M), C14:1, and SM C22:3 were reduced in CHD cases compared with the control specimens.

A similar series of analyses were performed using metabolites detected with the NMR platform. The 2-dimensional PCA (Figure 3, A) plot showed no separation between CHD cases and controls. Some clustering of cases relative to controls was observed on 2-dimensional PLS-DA analysis (Figure 3, B); however, the separation was not as clear as for the DI/LC-MS/MS analysis. Permutation analysis using 2000 resampling was performed to determine whether the observed separation was due to chance. The results of the permutation analysis showed that the probability that the observed separation or discrimination

FIGURE 1
2-D PCA and 2-D PLS-DA plots for DI/LC-MS/MS analysis



A, Two-dimensional PCA and **B,** 2-D PLS-DA plots (for DI/LC-MS/MS analysis) highlight the separation between controls in *green* and CHD cases in *red*.

CHD, congenital heart defect; *DI/LC-MS/MS*, direct injection liquid chromatography and tandem mass spectrometry; *PCA*, principal component analysis; *PLS-DA*, partial least squares discriminant analysis.

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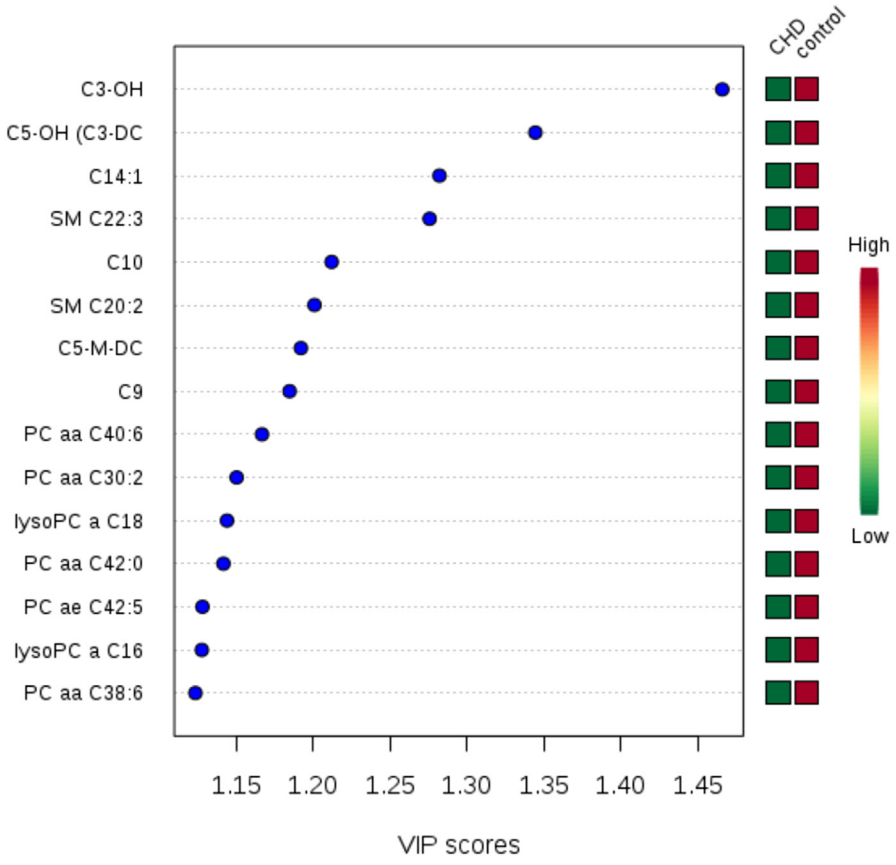
FIGURE 2
Variable importance in projection plot



VIP plot: the most discriminating metabolites are shown in descending order of importance. The color boxes indicate whether metabolite concentration is increased (*red*) or decreased (*green*) in controls vs CHD cases for DI/LC-MS/MS analysis.

CHD, congenital heart defect; *DI/LC-MS/MS*, direct injection liquid chromatography and tandem mass spectrometry; *VIP*, variable importance in projection.

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between severe CHD and controls was due to chance is relatively low ($P = .0175$). The corresponding VIP plot (figure not shown) showed acetone, ethanol, acetate, and pyruvate to be the 4 most discriminating metabolites using NMR analysis.

Using a logistic regression analysis, the individual probability of a fetus having CHD was calculated using 3 metabolites from the DI/LC-MS/MS–based metabolomics results: C3-OH, C5:1-DC, and hydroxytetradecadienylcarnitine (C14:2-OH) (Table 5). The logistic regression model for CHD vs control was represented by risk of CHD $= \ln(\pi/[1-\pi]) =$ $[-42.582 - 12.039 \log(\text{C3-OH}) + 3.194 \log(\text{C5:1-DC}) - 4.050 \log(\text{C14:2-OH})]$, where π is the probability of CHD. Table 5 shows the contribution of each of these DI/LC-MS/MS–based metabolites to the CHD prediction model. The ROC curve (Figure 4) indicates that this metabolite combination was a highly significant predictor of CHD: AUC, 0.981 (95% CI, 0.942–0.999).

The diagnostic performance of the algorithm is shown in Table 6. The sensitivity and specificity of the algorithms were statistically significant: 0.929 (95% CI, 0.833–1.00) and 0.932 (95% CI, 0.868–0.996), respectively. Permutation testing for the optimal model was performed using 2000 random samples and indicated a low probability that the diagnostic accuracy represented by the area under the ROC curve was due to chance, $P < .0005$.

We also looked at the performance of the algorithm using the NMR–based metabolites and also the metabolites combined with NT (Table 7). The ROC curve indicates that the metabolite combination (acetone and ethanol) was moderately diagnostic, AUC, 0.749 (95% CI, 0.628–0.854) with modest sensitivity (67.9%).

Using the NT measurement only, the following predictive equation for the CHD risk estimation was developed: $\ln(\pi/[1-\pi]) = -4.821 + 1.873$ NT, where π is the probability of CHD and NT was the nuchal translucency measurement. The AUC for this algorithm was 0.753 (95% CI, 0.616–0.867) with sensitivity (64.3%) and specificity (71.2%). NT contributed only modestly and did not significantly improve performance for either DI/LC-MS/MS and NMR-based metabolites (Tables 6 and 7). On a further analysis, CRL, ethnicity, BMI, or parity did not contribute significantly to CHD prediction using metabolites (results are not shown).

Analyses were also performed using both NMR and DI-mass spectrometry metabolites and NT ultrasound measurement for the CHD prediction (Figure 4). That prediction model was represented by $\ln(\pi/[1-\pi]) = -58.0591 + 2.1678$ NT $- 14.2494 \log(C_3\text{-OH}) + 2.9807 \log(\text{C5:1-DC}) - 4.6776 \log(\text{C14:2-OH})$, where π is the probability of CHD. Although we started out with both NMR and DI/LC-MS/MS–based metabolites, the logistic regression analysis selected only metabolites from the DI/LC-MS/MS assay (C3-OH, C5:1-DC, and C14:2-OH), none of the metabolites from the NMR assay were selected because those metabolites are less correlated with the classifiers. This metabolite combination showed the same results as in Table 6.

Comment

Using DI/LC-MS/MS and NMR metabolomic platforms, numerous metabolites were identified in maternal serum that distinguished chromosomally normal vs first-trimester CHD cases. The principal metabolite group identified was the

acylcarnitines. This chemical group represents intermediates involved in the transport and metabolism of fatty acids in the mitochondria. In addition, we demonstrated that the combination of a limited number of metabolites by themselves (eg, C3-OH, C5:1-DC, and C14:2-OH) appeared to be highly accurate predictors of CHD status. The sensitivity of this combination of metabolites was 92.9% at a specificity threshold of 93.2%. These values were highly statistically significant. C3-OH and C14:2-OH were reduced in CHD cases compared with the control specimens, whereas C5:1-DC was increased in CHD specimens.

Metabolites identified by the NMR platform alone provided only limited diagnostic accuracy. The combination of acetone and ethanol had a 67.9% sensitivity at 67.8% specificity. Nuchal translucency is an important marker in first-trimester aneuploidy risk determination.[3] Several studies have confirmed a modest correlation between translucency measurements in the first trimester and the risk of CHD.[20,21] We therefore looked at the combination of metabolite markers with NT measurement for the detection of CHD. Although a statistically significant predictor of CHD by itself, overall, there was no further benefit of adding NT measurements to the metabolite markers. There was an approximately 4% increase in sensitivity and specificity when NT measurement was added to the combination of acetone and ethanol in the case of NMR analysis; however, this increase was not statistically significant.

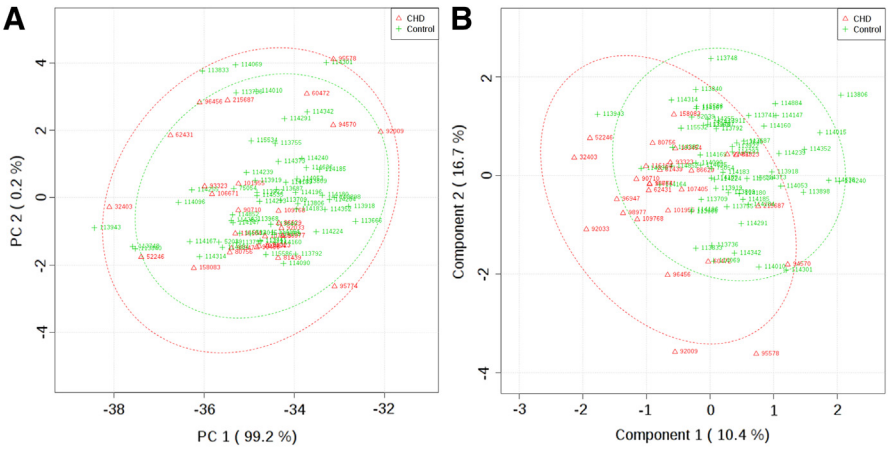
Reliable detection of CHD is the holy grail of prenatal screening. This directly reflects the importance of CHD. Congenital anomaly is the most important cause of infant death in the United States.[22] The prenatal detection of CHD has many theoretical benefits. Informing would-be parents of the presence of a fetal cardiac defect is critical for decision making, which involves a complex series of medical and personal choices.

Decisions such as transferring of care to an appropriate pregnancy specialist within the same institution or complete transfer to another institution with the appropriate high-risk obstetrical, newborn, and pediatric expertise often need to be made. Given the high rate of intervention and hospitalization in CHD cases,[23] there are significant social and financial implications to affected families. Prenatal diagnosis of CHD has been reported to improve medical costs. Data from the United States also found a greater than 10-fold increase in average newborn transportation costs when CHD was diagnosed postnatally compared with prenatal detection.[24]

Furthermore, there is suggestive evidence that at least in some types of CHD, prenatal diagnosis may improve newborn outcome.[25,26] Finally, although investigational, the increasing interest in fetal cardiac intervention for such lesions as aortic stenosis and hypoplastic left heart syndrome[27] creates another potentially powerful argument in favor of prenatal diagnosis, at least for cardiac anomalies that are amendable to such approaches.

Prenatal ultrasound remains the only tool currently available for the detection of CHD. Studies that are primarily from referral specialist centers report high diagnostic accuracy with specialized echocardiographic techniques such as spatiotemporal imagery correlation and

FIGURE 3
2-D PCA and 2-D PLS-DA plots for NMR analysis



A, Two-dimensional PCA and **B,** 2-D PLS-DA plots (for NMR analysis) highlight the separation between controls in *green* and CHD cases in *red*.

CHD, congenital heart defect; *NMR*, nuclear magnetic resonance; *PCA*, principal component analysis; *PLS-DA*, partial least squares discriminant analysis.

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TABLE 5
Logistic regression based optimal model for CHD detection: DI-MS metabolites only

Variable	Estimates (B)	SE	Z-value	Pr(>|z|)
(Intercept)	−42.582	18.604	−2.289	.022
C3-OH	−12.039	5.227	−2.303	.021
C5:1-DC	3.194	1.075	2.972	.003
C14:2-OH	−4.050	1.710	−2.369	.017

Logistic regression model is $\ln(\pi/[1-\pi]) = -42.582 - 12.039 \log(\text{C3-OH}) + 3.194 \log(\text{C5:1-DC}) - 4.050 \log(\text{C14:2-OH})$. where π is the probability of CHD.

CHD, congenital heart defect; *DI-MS*, direct injection mass spectrometry; *Pr(>|z|)*, 2-tailed *P* value used in testing the null hypothesis that the coefficient is 0 and z = z-value.

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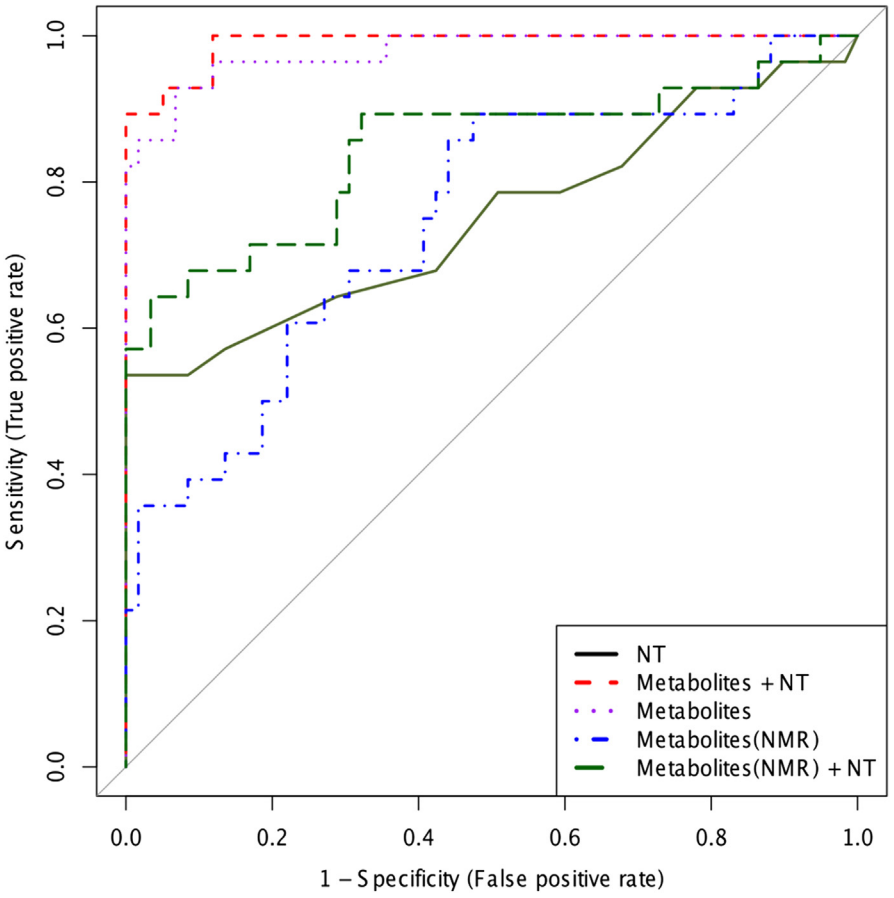
FIGURE 4
ROC comparison of all logistic regression models produced in this study



For NT, AUC = 0.753; for metabolites (DI-MS and NMR) plus NT, AUC = 0.992; for metabolites (DI-MS and NMR), AUC = 0.981; for metabolites (NMR) plus NT, AUC = 0.847; and for metabolites (NMR), AUC = 0.749 and for metabolites (DI-MS and NMR) and three metabolites used in the model (hydroxypropionylcarnitine, glutaconylcarnitine, and hydroxytetradecadienylcarnitine); for metabolites (NMR), acetone and ethanol.

AUC, area under the curve; *DI-MS*, direct injection mass spectromet; *NMR*, nuclear magnetic resonance; *NT*, nuchal translucency; *ROC*, receiver operating characteristic.

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TABLE 6
CHD prediction based on limited metabolite combinations: DI-mass spectrometry based metabolites

Metabolites/markers	AUC (95% CI)	Sensitivity, %	Specificity, %	*P* value[a]
Metabolites only[b]	0.981 (0.942–0.999)	92.9	93.2	< .001
Metabolites plus NT	0.992 (0.973–1.0)	92.9	93.2	< .001
NT only	0.753 (0.616–0.867)	64.3	71.2	.001

AUC, area under the curve; *CHD*, congenital heart defect; *CI*, confidence interval; *DI*, direct injection; *NT*, nuchal translucency.

[a] *P* value represents permutation test's *P* value; [b] Metabolites include hydroxypropionylcarnitine, glutaconylcarnitine, and hydroxytetradecadienylcarntine.

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combined cardiac anomaly detection.[28] Most population studies, however, paint a considerable less optimistic picture of achievable detection rates, even among groups with high (>90%) exposure to prenatal ultrasound.[29] In that study, the majority, close to 80% of nonchromosomal CHD cases, failed to be diagnosed prenatally in 29 population-based registries in 16 European countries.

The estimated current prenatal ultrasound screening practices in developed countries detected only 30-50% of fetal CHD cases. Despite the widely reported low CHD screening performance, few studies have, however, examined the reasons for such low diagnosis rates. Pinto et al[30] systematically reviewed the causes of the low CHD detection rate in their 10-year review of a statewide surveillance program in Utah. The CHD prenatal detection rate was only 39% overall. The main factors accounting for the failure to diagnose CHD prenatally was location in which the examination was performed (ie, hospital vs high-risk maternal fetal medicine office), the ultrasound interpreter (ie, obstetrician, radiologist, or maternal fetal medicine specialist), and the absence or presence of extracardiac anomalies.

A family history of CHD also increased the detection of cardiac anomalies, likely because of the identification of the patient as being at a sufficiently elevated risk with greater attention to detail on the ultrasound examination. Despite enhanced chances of diagnosis when a maternal-fetal medicine specialist performed the ultrasound, in 25% of such cases scanned in maternal-fetal medicine offices, the diagnosis was missed. There was universal availability of ultrasound in the study population.[30]

Other factors such as gestational age at the performance of ultrasound, maternal body habitus, and fetal lie are known to affect the chances of detecting a fetal cardiac anomalies. None of these limitations would appear to be relevant or significant if maternal biomarkers such as the examples reported in this preliminary study could be developed.

Although abnormality in metabolite levels in the folate pathway such as

homocysteine[14] and metabolites related to oxidative stress[15] have been previously reported, our study represents the first comprehensive untargeted metabolomics study for the prenatal prediction of CHD. The serum metabolomics profile of a first-trimester pregnant woman carrying a CHD fetus in this study found a significant elevation of acylcarnitines.

Carnitine (β-hydroxy-y-trimethylammonium butyrate) is a substance that plays a key role in the transfer of fatty acids into the mitochondria for metabolism and energy release.[31] Long-chain (multiple carbons) fatty acids bind with carnitine to form acycarnitines, which are transported into the mitochondria for sequential shortening, which occurs 2 carbons at a time. This process is associated with the generation of potential energy stored in adenosine triphosphate. During periods of starvation, these fatty acids constitute the main source of energy for skeletal muscle. Approximately 70% of myocardial energy is provided by mitochondrial fatty acid oxidation as described in previous text.[32]

Abnormality of folate metabolism has been linked to CHD in human[33] and animal[34] studies. Choline is an important nutrient that plays a role in lipid metabolism and in the formation of phosphotidyl choline for cell membrane synthesis. The 2 major roles of choline are for phospolipid synthesis and to serve as a methyl donor. Choline is oxidized to betaine in the mitochondria, and betaine serves as an actual methyl donor, which converts homocysteine to methionine. Increased levels of methionine are reportedly associated with a reduced risk of CHD, whereas elevated homocysteine is associated with increased CHD risk. Choline deficiency is also associated with increased lipid accumulation in the liver.[35] There is thus a plausible link between lipid and single carbon metabolism. Of note, in our data set, there was a reduction in carnitine levels in CHD vs normal pregnancies, providing further potential evidence of a metabolic disturbance in this pathway.

Disturbances of phosphatidyl choline metabolism is a prominent feature of several cancers including breast cancer.[36] Cancer is a disorder characterized by rapid cell growth, division, and apoptosis. Given the critical role of phosphotidylcholine in cell membranes, the effect of disturbance in choline metabolism is understandable. Organogenesis in the embryonic period has obvious similarities to cancer. It is therefore possible that in CHD, abnormalities of tissue remodeling, which affect the rate of cell membrane synthesis and destruction, may be manifesting as the abnormality of the choline and phosphotidylcholine metabolism.

In summary, we identified evidence of extensive phosphatidyl-choline and lipid abnormalities in the first-trimester serum of pregnant women with CHD fetuses. Some of these metabolic abnormalities such as the disturbance of carnitine levels and therefore lipid synthesis could plausibly be tied to aberrations of single-carbon metabolism through choline. There is already extensive evidence of an association with altered homocysteine and methionine levels and the development of CHD.

Our study has some limitations. First, this is a small pilot study with limited demographic variation. The conclusions derived herein may not apply either to a larger or a substantially different population. Of note, we found no correlation at this time between gestational age, maternal demographic characteristics such as ethnicity and BMI, and the metabolite levels in this study. The screening performance found in this study therefore cannot be extrapolated to the general population. The markers identified provide preliminary evidence of a role of metabolomics for the development of biomarkers for CHD detection. Despite the observed association with CHD, we cannot at this time make any claims regarding clinical utility.

It should be noted that metabolomic analysis is technologically demanding and requires significant expertise. Of critical importance is the meticulous preparation and early freezing of specimens that are being stored for subsequent analyses. Careless handling of specimens and lack of attention to detail could significantly affect the results. This is of inestimable importance for others planning to perform metabolomic studies. However, many of the individual metabolites have been assayed for years using conventional and widely available laboratory technologies, for example, acetone. This suggests that it could turn out to be relatively easy to transfer many of these metabolites to general usage.

In conclusion, we have reported a significant disturbance in lipid including phosphatidyl-choline and various sphingolipids and choline metabolism in the first-trimester serum of women carrying CHD fetuses. This appears to be a new finding because we could not identify prior such publications in the literature. Furthermore, in the first step toward developing biomarkers for CHD prediction, a limited number of metabolites appear to have significant diagnostic accuracy for the biochemical prediction of CHD in the first-trimester fetus. It is too early to be able to extrapolate these results to other populations, however. ■

TABLE 7
NMR-based prediction of CHD

Metabolites/markers	AUC (95% CI)	Sensitivity, %	Specificity, %	*P* value[a]
Metabolites only[b]	0.749 (0.628–0.854)	67.9	67.8	.002
Metabolites plus NT	0.847 (0.729–0.937)	71.4	71.2	< .001
NT only	0.753 (0.616–0.867)	64.3	71.2	.001

AUC, area under the curve; *CHD*, congenital heart defect; *CI*, confidence interval; *DI*, direct injection; *NMR*, nuclear magnetic resonance; *NT*, nuchal translucency.

[a] *P* value represents permutation test's *P* value; [b] Metabolites include acetone and ethanol.

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