OBSTETRICS

Metabolomic analysis for first-trimester Down syndrome prediction

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OBJECTIVE: The objective of the study was to perform first-trimester maternal serum metabolomic analysis and compare the results in aneuploid vs Down syndrome (DS) pregnancies.

STUDY DESIGN: This was a case-control study of pregnancies between 11+0 and 13+6 weeks. There were 30 DS cases and 60 controls in which first-trimester maternal serum was analyzed. Nuclear magnetic resonance-based metabolomic analysis was performed for DS prediction.

RESULTS: Concentrations of 11 metabolites were significantly different in the serum of DS pregnancies. The combination of 3-hydroxyisovalerate, 3-hydroxybuterate, and maternal age had a 51.9% sensitivity at 1.9% false-positive rate for DS detection. One multimarker algorithm had 70% sensitivity at 1.7% false-positive rate. Novel markers such as 3-hydroxybutyrate, involved in brain growth and myelination, and 2-hydroxybutyrate, involved in the defense against oxidative stress, were found to be abnormal.

CONCLUSION: The study reports novel metabolomic markers for the first-trimester prediction of fetal DS. Metabolomics provided insights into the cellular dysfunction in DS.

Key words: Down syndrome screening, metabolomics

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Metabolites are low-molecular-weight organic and inorganic chemicals that are the substrates, intermediates, and byproducts of enzyme-mediated biochemical reactions in the cell.[1] The molecules include but are not limited to carbohydrates, amino acids, peptides, nucleic acids, organic acids, vitamins, lipids, and other biological species.[2] Metabolomics refers to the systematic identification and quantification of these small (less than 1500 Da) molecules.

★ EDITORS' CHOICE ★

Advances in the development of analytic platforms such as nuclear magnetic resonance (NMR) and high-performance liquid chromatography–mass spectrometry[3] and in statistical programs for analysis of the large volume of data generated in metabolomic studies[4] have fueled the rapidly growing interest in this field.

Metabolites form the building blocks of genes, ribonucleic acid transcripts, and proteins. For these and other reasons, metabolomics is thought to have the potential to provide more fundamental and global information than genomics, proteomics, and transcriptomics and to more precisely reflect the phenotype of the cell. The human serum metabolome has now been extensively characterized. A total of 4229 confirmed and highly probable compounds have recently been reported.[5]

One practical application of metabolomics has been in the development of biomarkers for disparate and complex disorders such as schizophrenia,[6] meningitis,[7] and colon cancer.[8] To date, there has been only very limited use of metabolomics in obstetrics.

Routine screening for Down syndrome (DS) in pregnancy has been the standard of clinical care in the United States[9] and other developed countries.[10] The identification of new biomarkers for DS screening continues to be an area of intense research interest.[11]

The purpose of this study was to determine whether metabolomic markers in maternal blood can distinguish DS from normal pregnancies during the first trimester. We also performed a preliminary evaluation of the diagnostic accuracy of some of these markers.

Materials and Methods

This was a case-control study drawn from a large prospective study to identify potential biomarkers of pregnancy complications in women attending for their routine first hospital visit in pregnancy at King's College Hospital (London, UK) from March 2003 to February 2009. In this visit, which is held at 11^{+0}-13^{+6} weeks of gesta-

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TABLE 1
Demographic and other characteristics of the study population

Parameter	Down syndrome	Euploid control	*P* value
Number of cases	30	60	—
Maternal age (y), mean (SD)	37.2 (3.9)	30.5 (7.1)	< .01
Racial origin, n (%)			.012
White	27 (90)	33 (55.9)	
Black	2 (6.7)	19 (32.2)	
Asian	1 (3.3)	4 (6.8)	
Mixed	0 (0)	3 (5.1)	
Nullipara, n (%)	8 (26.7)	25 (42.4)	.11
Weight (kg), mean (SD)	66.3 (14.9)	67.0 (15.0)	.83
Medical disorder present	6 (20)	5 (8.5)	.11
Crown-rump length (mm), mean (SD)	67.2 (7.4)	64.8 (8.7)	.19

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tion, all women have combined screening for aneuploidies.[12] Maternal demographic characteristics, ultrasononographic measurements, and biochemical results were recorded in a computer database. Karyotype results and details on pregnancy outcomes were added into the database as soon as they became available. All cases of trisomy 21 had a fetal karyotype confirmed following a chorionic villus sampling. The controls were selected from women delivering live-born neonates at full term with a normal karyotype or a normal phenotypic examination.

Written informed consent was obtained from the women agreeing to participate in the study, which was approved by the King's College Hospital Ethics Committee. Maternal venous blood collected in plain BD vacutainer tubes (Becton Dickinson UK Limited, Oxfordshire, UK) was processed within 15 minutes of blood collection and centrifuged at 3000 rpm for 10 minutes to separate serum from packed cells. The maternal serum obtained after centrifugation was then divided into 0.5 mL aliquots and stored at −80°C until subsequent analysis. None of the samples were previously thawed and refrozen.

An aliquot of their serum were used for the measurement of free β-human chorionic gonadotropin (hCG) and pregnancy associated plasma protein A (PAPP-A). The serum samples were transported to the laboratory (Edmonton, Canada) for metabolomic testing by air courier on dry ice and maintained in a frozen state.

The case-control study population consisted of 30 cases with fetal trisomy 21 and 60 euploid controls. Each case of fetal trisomy 21 was randomly chosen from archival specimens and was matched with 2 euploid controls with blood collected within 1 week of each other. None of the samples in the case-control study were previously thawed and refrozen or used in any pilot study.

NMR spectroscopy was the analytic platform used for metabolomic analysis. NMR is very quantitative but is not a particularly sensitive approach for metabolomics. Typically only 35-45 compounds can be identified or selected from a serum NMR spectrum on routine analysis.[5] We have previously described in detail the sample preparation, spectroscopic methods, and statistical analyses used.[13] A 500 MHz Varian Inova NMR spectrometer was used to collect the spectra of all serum samples. Overall, 40 serum metabolites were identified, quantified, and compared in each DS and normal case using the NMR Chenomx NMR Suite 7.1 (Edmonton, Canada), a commercial software package for spectral identification and quantification of metabolites. It is from this set of 40 detectable metabolites that the DS metabolite signature was determined. Standard statistical techniques recommended for metabolomic analysis including normalization, principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA) were used.[14,15]

Data normalization is important to reduce systematic bias or technical variation and to improve statistical robustness. This was accomplished using log normalization. PCA is a multivariate technique[16] that identifies the uncorrelated variables or metabolites (principal components) that account for the largest variance or difference between the study and control groups. The metabolites are ordered based on their contribution to the variance between the 2 groups. In the PCA plot, the first principal component is represented on the X-axis and the second most important on the Y-axis (with the third principal component on the Z-axis in a 3-dimensional plot). The minimum number of principal components that when combined together accounts for the highest percentage of the total variance between groups is sought. Clustering of the data points on the PCA plot provides visual evidence that the study and control groups can be discriminated by measuring the principal components.

PLS-DA was used to further enhance the discrimination between study and control groups. With PLS-DA analysis, the principal components are rotated to find the metabolites most responsible for separating cases from controls[14] PLS-DA therefore improves separation between the groups. PLS-DA is typically performed after separation is demonstrated on PCA. There is a risk in PLS-DA analysis of observing separation between controls and study group that might not be statistically significant. To minimize this risk, permutation testing is performed with random relabeling of the metabolomic data and rerunning of the PLS-DA analysis. After 2000 repetitions of the relabeling process, the *P* values of the probability that the observed separation might be due to chance were calculated. PCA and PLS-DA analyses were performed with a MetaboAnalyst computer program.[17]

A variable importance in projection (VIP) plot was also constructed. This is a graph of the relative contributions of individual metabolites to the variance between the 2 groups.[4] The higher the VIP value (X- and Y-axis), the greater is the

individual contribution of that metabolite to group separation.

Statistical analysis

Mean and SD metabolite concentrations were compared between groups. Logistic regression analysis was performed using a limited number of metabolites only and metabolites plus maternal age for the prediction of DS. Based on these regression equations, receiver operating characteristic (ROC) curves plotting sensitivity against false-positive rate values for DS prediction were generated. Correlation analysis between the most important metabolites based on VIP and maternal age and ethnicity, which are commonly used to standardize DS biomarkers, were performed. Kolmogorov Smirnov and Shapiro-Wilk tests of normal distribution were performed. Power analysis indicated that a minimum of 17 cases and 34 controls were needed in each group to have 80% power for a 2-sided $P < .05$. The statistical significance was set at $P < .05$.

Apart from standard statistical analyses, genetic programming (GP) was utilized to identify significant metabolite and other predictors of DS. The diagnostic accuracies of various marker combinations were evaluated with GP. These included demographic and clinical parameters (maternal age, ethnicity, weight, parity, tobacco use, and the presence of medical disorders along with the metabolites). Evolutionary algorithms, including genetic algorithms, and their use in metabolomic analyses have been the subjects of prior reviews.[18,19] Genetic computing is a branch of GP. We had previously published an explanation of this approach.[13] Briefly, GP uses the principles of evolutionary genetics, namely selection, recombination, and mutation. Rules are generated by which the optimal combinations of predictive markers are identified. GP does not suffer from the limitations of conventional statistical analysis (eg, requiring normality of the data distribution and handling the absence of data points) and is equally useful for evaluating extremely large data collections or a small volume of data and has been claimed to be superior to standard statistical analyses. GP analysis was performed by The GMAX

TABLE 2
Maternal serum metabolite concentration

Metabolite	Down syndrome Mean (SD)	Euploid controls Mean (SD)	*P* value
Number of cases	30	60	—
2-hydroxybutyrate	23.1 (14.1)	15.0 (7.4)	.008
3-hydroxybutyrate	62.7 (85.0)	21.0 (19.4)	.018
2-hydroxyisovalerate	6.9 (4.0)	4.1 (2.8)	.002
Acetamide	9.2 (5.5)	6.4 (4.9)	.028
Acetate	27.5 (17.6)	30.1 (22.6)	.576
Acetoacetate	23.5 (24.3)	13.9 (7.3)	.054
Acetone	15.6 (8.7)	10.5 (3.3)	.007
Alanine	247.2 (89.1)	231.9 (87.3)	.46
Arginine	125.6 (37.3)	132.7 (50.7)	.516
Asparagine	27.3 (9.6)	25.6 (10.3)	.48
Betaine	25.4 (5.5)	27.0 (8.9)	.411
Carnitine	25.5 (9.7)	19.5 (8.3)	.004
Choline	31.6 (113.2)	52.7 (143.9)	.507
Citrate	57.2 (18.7)	51.9 (16.5)	.208
Creatine	37.4 (13.3)	34.4 (11.8)	.308
Creatinine	50.1 (12.6)	48.0 (11.9)	.466
Dimethylamine	33.6 (19.0)	49.1 (30.6)	.019
Ethanol	14.4 (10.4)	14.1 (8.4)	.859
Formate	3479.9 (628.4)	3496.6 (620.1)	.90
Glucose	245.5 (96.0)	217.3 (73.8)	.148
Glutamine	150.4 (128.0)	174.3 (216.4)	.60
Glycerol	205.7 (57.44)	203.1 (79.9)	.88
Glycine	5.1 (2.5)	4.1 (1.7)	.056
Isobutyrate	38.0 (14.0)	35.2 (15.4)	.434
Isopropanol	4.7 (4.2)	6.1 (5.3)	.229
Lactate	1172.1 (683.4)	884.7 (369.2)	< .05
Leucine	67.3 (34.6)	60.0 (46.9)	.87
Malonate	13.7 (7.1)	15.8 (9.7)	.336
Methionine	25.0 (6.9)	32.6 (11.5)	< .001
Ornithine	30.5 (12.1)	26.0 (10.8)	.10
Phenylalanine	55.4 (27.0)	46.2 (30.8)	.191
Proline	142.2 (60.4)	134.0 (52.4)	.53
Propyl-glycol	7.0 (2.9)	6.6 (1.9)	.49
Pyruvate	77.9 (32.4)	61.0 (26.4)	.014
Serine	135.3 (45.2)	148.4 (52.2)	.29
Succinate	5.3 (5.6)	5.6 (12.5)	.91
Threonine	123.3 (31.8)	119.3 (45.2)	.68
Tyrosine	50.5 (15.4)	54.1 (20.7)	.436
Valine	117.1 (33.4)	114.8 (34.9)	.774
L-methylthistidine	40.4 (12.3)	32.7 (13.7)	.016

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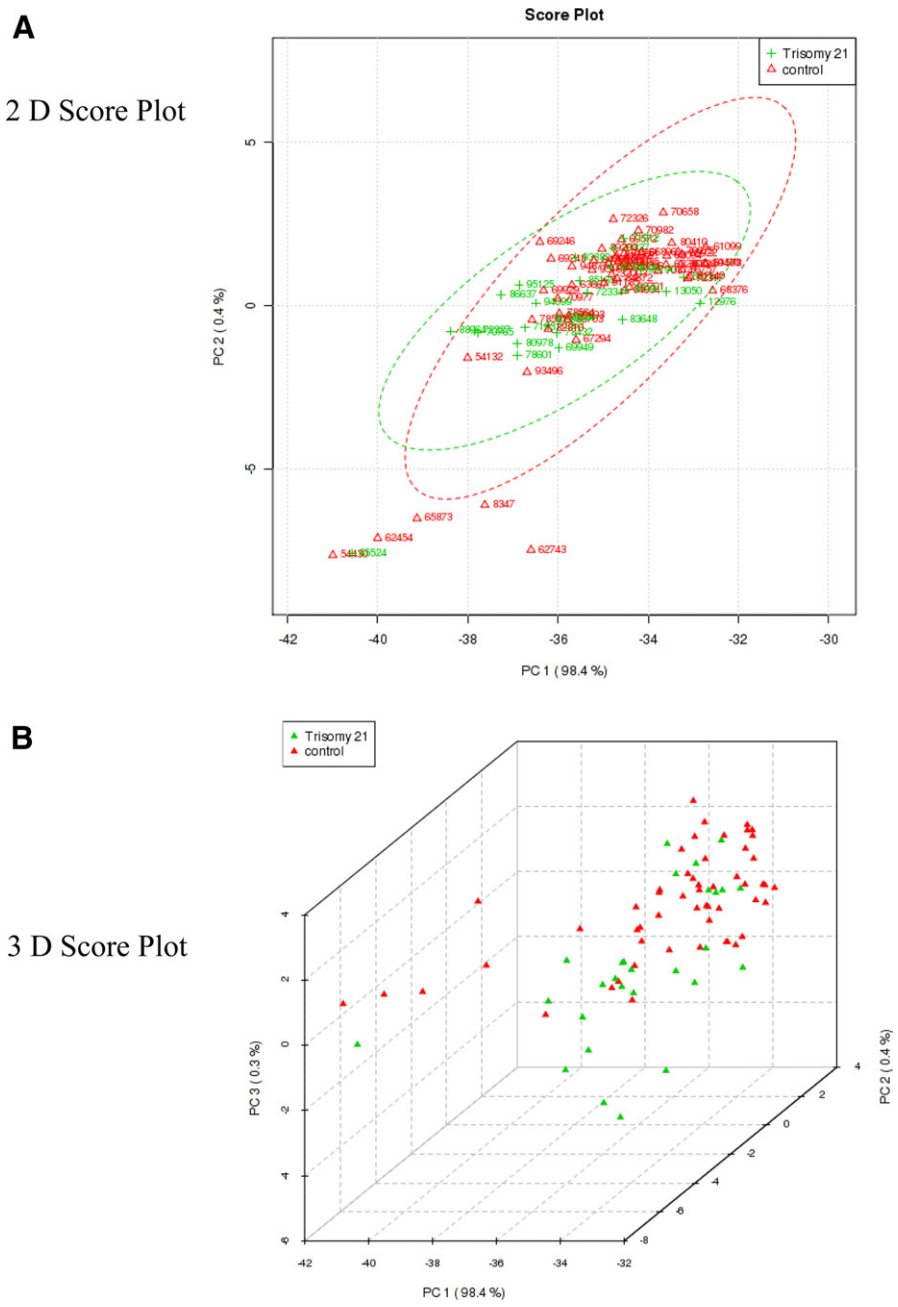
FIGURE 1
PCA plot: DS vs normal



A, Two-dimensional score plot. **B,** Three-dimensional score plot.

DS, Down syndrome; *PCA,* principal component analysis.

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computer program, version 11.9.23 (www.Thegmax.com).

RESULTS

Results were obtained for 30 DS cases and 60 controls. The demographic and clinical characteristics of DS cases and controls are shown in Table 1. As expected, DS mothers were significantly older than control mothers. In addition, there were racial differences between the 2 groups. In Table 2, the serum metabolite concentrations are compared between cases and controls. A total of 11 metabolites had significantly different concentrations between the DS and normal groups, whereas the differences were of borderline significance in the case of 2 metabolites. Figure 1, A and B, show 2- and 3-dimensional PCA plots. The contributions of the principal components to the variance between DS and normal groups are shown on the X- and Y- axes on the 2-dimensional PCA plot (and Z-axis in the case of the 3-dimensional plot). There is visual evidence of separation between the 2 groups. The corresponding PLS-DA plots are shown in Figure 2, A and B.

Figure 3 is a VIP plot that indicates that 3-hydroxybutyrate, 3-hydroxyisovalerate, and 2-hydroxybutyrate are the most discriminating metabolites. On the right of the VIP plot is a heat map. In the trisomy 21 column, red indicates that the concentration of the particular metabolite was increased in the DS group compared with normal, whereas green indicates reduced metabolite concentration. As shown in Figure 3, a total of 2000 permutations were performed and the separation observed between the groups was highly statistically significant (P = .0025). Using standard regression models based on a limited number of metabolites only as shown in Table 3, 48.1% DS detection rate was achieved at a 3.1% false-positive rate. Furthermore, a specificity rate of 100% (0% false-positive rate) and a detection rate of 40.7% was observed with this particular algorithm.

The screening performance when metabolites were combined with maternal age is also shown in Table 3. A 51.9% detection rate was achieved with just a 1.9% false-positive rate. The areas under the ROC curves for the 2 approaches, metabolites alone and metabolites plus maternal age, are shown in Table 4. In the normal group, maternal age appeared to correlate with the 2-hydroxybutyrate concentrations (r = 0.335, P = .13). Neither 2-hydroxybutyrate nor 3-hydroxyisovalerate concentrations appeared to significantly correlate with maternal age in the normal group. There was no significant correlation between any of the 3 metabolite concentrations and age in the DS group.

Direct comparisons between the euploid white (n = 33) and black (n = 19) serum specimens did not show a signifi-

cant difference in the concentrations of any of the 40 metabolites (t test for equality of means, all $P \geq .05$). Similarly, there were no differences in the frequency of smoking, another potential confounder, between whites and blacks (6.1% and 5.3% respectively, $P = 1.00$).

Gestational age did not appear to correlate significantly with metabolite levels.

GP was also used to develop 2 algorithms that were based on metabolites only (Table 5). Models using both the minimum (parsimonious) and an expanded number of metabolites were evaluated. In Table 6, expanded screening models combining metabolite, crown-rump length (CRL) measurements, and maternal demographic characteristics were similarly evaluated based on GP. Statistically significant diagnostic accuracy for the detection of DS was achieved using different biomarker combinations including the metabolite-only prediction. Maternal race did not appear to significantly contribute to DS prediction in the GP models (Table 6). Repeat analysis after removal of 6 outlier specimens with unusually high concentrations of most metabolites resulted in alteration of the order of the most significant discriminating metabolites in the VIP plot (not shown). Among the 3 most discriminating metabolites, succinate replaced 2-hydroxybutyrate as the third most important metabolite in the VIP analysis. The order of some of the less discriminating metabolites was also slightly changed.

FIGURE 2
PLS-DA plot: DS vs normal

A, Two-dimensional score plot. **B,** Three-dimensional score plot.
DS, Down syndrome; *PLS-DA,* partial least squares discriminant analysis.
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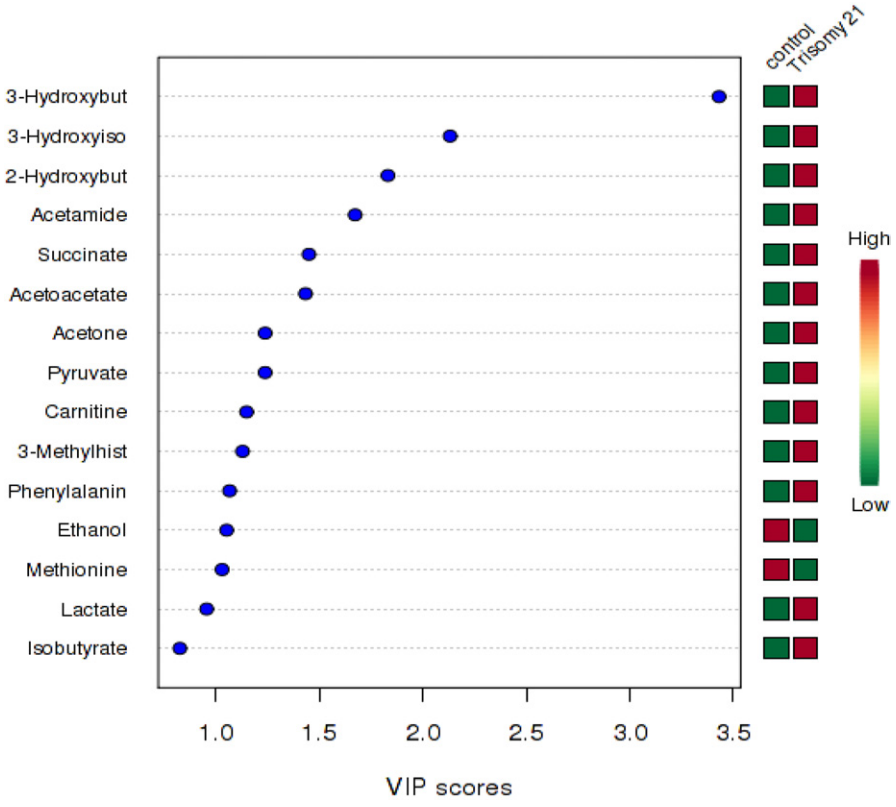
COMMENT

In a study using NMR-based metabolomics, we found significant changes in a number of metabolites in the maternal serum during the first trimester of pregnancies complicated with a DS fetus. Using logistic regression analysis, the detection rate of DS was 48.1% at a false-positive rate of less than 2%. In addition to conventional statistical analyses, multivariate statistical and graphic approaches designed to interpret metabolomic data (such as PCA and PLS-DA analysis) confirmed significant variance between DS and normal cases. Further analysis was performed using GP, which is thought to have advantages over standard statistical analysis (www.TheGmax.com). Based on GP using either a limited or parsimonious algorithm or an expanded panel of biomarkers, metabolites by themselves, or combined with other markers, were found to be significant predictors of first trimester DS cases.

The finding of a profound alternation of the maternal serum metabolome in the first trimester of DS pregnancies is not surprising. The disturbance in trophoblast function leading to changes in the maternal serum concentrations of glycoproteins such as hCG and PAPP-A in addition to non–trophoblast-related biochemical markers is well known.[20] Combined ultrasound and biochemical screening forms the basis of current first-trimester DS screening protocols.[11,21]

An advantage of metabolomics is that it is hypothesis generating. Thus, its deployment is not limited by a lack of prior understanding of disease pathogenesis. Rather, metabolomic data can help to develop an understanding of disease mechanisms,

FIGURE 3
VIP plot



Asterisk indicates 2000 permutations performed ($P = .0025$).

VIP, variable importance in projection.

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whereas this is not previously known or well understood.

Based on VIP analysis, 3-hydroxybutyrate, 3-hydroxyisovalerate and 2-hydropoxybutyrate appeared to be the most discriminating metabolites for distinguishing DS cases from normal controls. We searched the Human Metabolomics Database (www.hmdb.com) to review the function of these metabolites in normal and/or disease states and to see what plausible role they might play in the DS pathology. 3-hydroxybutyrate is a ketone body, similar to acetoacetate and acetone. It is an indispensable source of energy for extrahepatic tissues such as the brain. It is an important substrate for the synthesis of phospholipids and sphingolipids that are required for brain growth and myelination. 3-hydroxybutyrate and other ketone bodies are utilized in cerebroside synthesis during the period of active myelination in the brain. Brain studies from 17 weeks to childhood have confirmed delayed myelination in DS compared with normal controls.[22] In general, decreased myelination is associated with mental retardation.

The compound 3-hydroxyisovalerate is a metabolite that is usually excreted in urine. Multiple enzyme deficiencies including carboxylase and biotinidase deficiencies result in elevated 3- hydroxyisovalerate levels. This compound is itself a widely used biomarker for biotinidase deficiency. In addition, prolonged anticonvulsant use such as phenytoin can be associated with elevation of 3-hydroxybtyrate. Biotin (vitamin B7) deficiency results from failure of biotin release (a biotinidase function) from dietary proteins and can result in hypotonia, learning disability, seizure disorders, and brain atrophy,[23] all of which are features of DS.

Finally, we looked at 2-hydroxybutryate. It is an organic acid that is elevated in states of oxidative stress. During oxidative stress there is a limited supply of L-cysteine, the substrate for glutathione (antioxidant) synthesis. Under these circumstances, homocysteine, which is converted to methionine in the remethylation pathway during folate metabolism, gets diverted to the transsulfuration pathway for the synthesis of cystathionine. Cystathionine is then converted to cysteine by the enzyme cystathione β-synthase (CBS) and used for the synthesis of the antioxidant glutathione. 2-hydroxybutyrate is released as a byproduct in the cleavage of cystathionine. Metabolic disorders affecting neonatal brain development such as lactic acidosis are often associated with elevated 2-hydroxybytrate levels (www.hmdb.ca).

The association between 2-hydroxybutyrate, oxidative stress, and single carbon metabolism is particularly interesting in the context of what is currently known about brain dysfunction in DS. DS is known to be accompanied by abnormalities of several enzymes involved in folate and 1 carbon metabolism.[24] CBS is overexpressed in the brains of DS patients.[25] Indeed, the CBS gene is located at 21q 22.3 (gene database: www.ncbi.nlm.nih.gov). Oxidative stress is thought to be 1 of the most likely causes of neurotoxicity in DS.[26]

Morphological abnormalities in the brains of DS individuals generally become identifiable after fetal life. Obvious morphological changes, however, ap-

TABLE 3
First-trimester Down syndrome prediction: logistic regression

Models	Sensitivity, %	False-positive rate, %
Metabolites only[a]	48.1	3.1
Metabolites and maternal age[b]	51.9	1.9

[a] 3-hydorxybuterate and 3-hydroxyisovalerate; [b] 3-hydroxybutyrate, 3-hydroxyisovalerate, and maternal age.

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pear to be preceded by a host of biochemical changes in the DS fetal brain and have been documented in numerous studies.[27] Examples include elevated superoxide dismutase (SOD), a known antioxidant, presumably in response to the increased oxidative stress. Not surprisingly, the SOD gene is also coded on chromosome 21. It is possible that the main source of these metabolomic changes noted in the maternal blood in this study is the placenta, a fetal tissue, rather than the actual first trimester fetal brain or other fetal tissues. For example, trophoblast tissue from DS pregnancies demonstrate comparable biochemical response as described in previous text in the fetal brain such as elevated SOD gene expression and SOD protein levels.

[28] Overall, this preliminary analysis of the metabolomic profile of maternal serum in DS pregnancies appears suggestive of a metabolic environment conducive to a disturbance in the fetal central nervous system development. Metabolomics could improve our understanding of the pathologic consequences of the DS genotype on brain development and facilitate prenatal mitigating therapy.

A recent publication reported high diagnostic accuracy for the detection of trisomy 21 and trisomy 18 using cell-free fetal deoxyribonucleic acid (cfDNA) in maternal first-trimester blood.[29] In that case-control study, 100% sensitivity and 98% specificity were reported respectively for the detection of trisomy 21 and trisomy 18 at a specificity of 100%. The authors emphasized, however, that in addition to DS screening, mounting evidence of the value of combined sonographic and biochemical first-trimester markers for the prediction of other more common pregnancy disorders such as gestational diabetes, preeclampsia, stillbirth, and fetal growth restriction suggest that screening approaches using sonographic and biochemical markers will become more widespread rather than be replaced by molecular testing for aneuploidy screening.[29]

Based on this reasoning, molecular cfDNA testing is being considered as a second-stage test for patients who are DS screen positive based on conventional biochemical and sonographic screening.

TABLE 4
Model prediction based on area under the ROC curve: logistic regression approach

Model	AUC	95% CI	*P* value
Metabolites only[a]	0.757	0.627–0.89	< .001
Metabolites and maternal age[b]	0.862	0.778–0.946	< .001

AUC, area under the ROC; *CI*, confidence interval; *ROC*, receiver operating characteristic.

[a] 3-hydroxybutyrate and 3-hydroxyisovalerate; [b] 3-hydroxybutyrate, 3-hydroxyisovalerate, and maternal age.

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TABLE 5
Down syndrome detection, metabolites only (genetic computing)

Model	AUC	Sensitivity, %	Specificity, %
Parsimonious[a]	0.775	75	86.2
Expanded[b]	0.879	56.7	94.9

AUC, area under the receiver operating characteristic curve ($P < .001$).

[a] 3-hydroxyisovalerate, 2-hydroxybutrate, and 3-hydroxybutrate; [b] 3-hydroxybutrate, carnitine, 3-hydroxyisovalerate, creatine, methylhistidine, methionine, and creatinine.

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TABLE 6
Down syndrome screening: metabolites and other predictors (genetic computing)

Model	AUC	Sensitivity	Specificity
Parsimonious[a]	0.807	60	85.0
Expanded[b]	0.914	70	98.3

AUC, area under the receiver operating characteristic curve ($P < .001$).

[a] Maternal age, propylene glycol, and acetone; [b] Maternal age, creatinine, 3-hydroxybutrate, creatine, glucose, crown-rump length, and parity.

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It is reasonable to anticipate that further advances that could lead to improvement in biochemical screening accuracy will occur as has been illustrated in this manuscript. Our pilot study did not ascertain the impact on diagnostic accuracy when these metabolites were combined with traditional markers such as hCG, PAPP-A, and nuchal translucency. It is plausible that such a strategy could further improve the DS sensitivity and specificity above conventional markers.

In summary, we report multiple metabolomic markers of fetal DS in first-trimester maternal blood. Some of these metabolites are known to be associated with oxidative stress, poor myelination, and neurotoxicity of the brain in DS individuals. ■

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