Altered Metabolism of Mothers of Young Children with Autism Spectrum Disorder: A Case Control Study

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Abstract

Background: Previous research studies have demonstrated abnormalities in the metabolism of mothers of young children with autism.

Method: Metabolic analysis was performed on blood samples from 30 mothers of young children with Autism Spectrum Disorder (ASD-M) and from 29 mothers of young typically-developing children (TD-M). Targeted metabolic analysis focusing on the folate one-carbon metabolism (FOCM) and the transsulfuration pathway (TS) as well as broad metabolic analysis were performed. Statistical analysis of the data involved both univariate and multivariate statistical methods.

Results: Univariate analysis revealed significant differences in 5 metabolites from the folate one-carbon metabolism and the transsulfuration pathway and differences in an additional 48 metabolites identified by broad metabolic analysis, including lower levels of many carnitine-conjugated molecules. Multivariate analysis with leave one-out cross validation allowed classification of samples as belonging to one of the two groups with 93% sensitivity and 97% specificity with five metabolites. Furthermore, each of these five metabolites correlated with 8-15 other metabolites indicating that there are five clusters of correlated metabolites. In fact, all but 5 of the 50 metabolites with the highest area under the receiver operating characteristic curve were associated with the five identified groups. Many of the abnormalities appear linked to low levels of folate, vitamin B12, and carnitine-conjugated molecules.

Conclusions: Mothers of children with ASD have many significantly different metabolite levels compared to mothers of typically developing children at 2-5 years after birth.

Keywords: Autism, metabolic profile, Fisher Discriminant Analysis, Logistic Regression, metabolomics, mothers

Background

Autism spectrum disorder (ASD) involves a combination of abnormal social communication, stereotyped behaviors, and restricted interests (1). ASD is assumed to be caused by complex interactions between genetic and environmental factors, both of which can affect metabolism. Previous studies have revealed significant abnormalities in the folate-one carbon metabolism and the transsulfuration pathways of children with ASD (2-5) and their mothers (6, 7, 8), resulting in decreased methylation capability, decreased glutathione levels, and increased oxidative stress. Furthermore, the presence of mutations in the *MTHFR* gene (A1298C and C667T) was found to be associated with increased risk of ASD (9). Additionally, levels of prenatal vitamins taken during pregnancy that include B12 and folate are associated with a decreased ASD risk (10), suggesting an association of metabolite levels of the folate one-carbon metabolism (FOCM) and the transsulfuration pathway (TS) pathways with ASD. Studies found that maternal gene variants in the one-carbon metabolism pathway were associated with increased ASD risk when there was no or only low levels of periconceptional prenatal vitamin intake (11, 12).

Additional metabolic differences may also be present in mothers of children with ASD, but there has been relatively little investigation of their metabolic state. A more comprehensive understanding of metabolites and metabolic pathways of mothers of children with ASD may lead to a better understanding of the etiology of ASD and provide some insights for evaluating pre-conception risk and/or risk during pregnancy. For example, currently, the general risk of having a child with ASD in the US is approximately 1.9% (13), however, the recurrence risk increases to approximately 19% if the mother already has a child diagnosed with ASD (14).

This paper focuses on analyzing the metabolic profile of mothers of young children with ASD and mothers of typically developing children, 2-5 years after birth. Measurements were conducted with whole blood to provide information on both intra-cellular and extra-cellular metabolism. This study was limited to women who were not taking folate, B12, or multi-vitamin/ mineral supplements during the 2 months prior to sample collection, in order to minimize the effect of supplements on metabolism. The study includes assessments of many different aspects of metabolism, including analysis of amino acids, peptides, carbohydrates, lipids, nucleotides, Kreb's cycle, vitamins/co-factors, and xenobiotics. This work is part of a larger study, the ASU-Mayo Pilot Study of Young Children with ASD and their Mothers (AMPSYCAM).

Although it would be ideal to have biological samples obtained during conception, pregnancy, lactation, and infancy, this would represent a significant hurdle for study design. Instead, this pilot study focuses on 2-5 years after birth to provide preliminary insight into metabolic abnormalities that currently exist. Results from this study provide the motivation for larger future studies to validate the findings and potentially to expand the time horizon to include the time during conception/pregnancy/lactation.

Methods

Study Design and Sample Collection and Analysis

IRB Approval and Consent. This study was approved by the IRB of Mayo Clinic-Arizona and the IRB of Arizona State University. All parents signed informed consent forms after the study was explained to them.

Advertising. The study advertisement was emailed to several thousand ASD families on the email lists of the ASU Autism/Asperger's Research Program and the Zoowalk for Autism Research. Other local autism groups such as the Autism Society of Greater Phoenix also helped advertise the study. Finally, participants were invited to share the study advertisement with their network of friends.

Participants. The inclusion criteria were:

1) Mother of a child 2-5 years of age

2) Child has ASD or has typical development (TD) including both neurological and physical development

3) ASD diagnosis verified by the Autism Diagnostic Interview-Revised (ADI-R)

The exclusion criteria were:

1) Currently taking a vitamin/mineral supplement containing folic acid and/or vitamin B12

2) Pregnant or planning to become pregnant in the next six months

Thirty mothers who have a child with ASD (ASD-M) and twenty-nine mothers who have TD children (TD-M) were recruited for this study. All mothers in the ASD-M group had a child previously diagnosed with ASD and their diagnosis was confirmed using the ADI-R. The ADI-R is a 2-hour structured parent interview and is one of the primary tools used for clinical and research diagnosis of ASD. All of the ADI-R interviews were conducted by Elena L. Pollard, who is a certified rater on the ADI-R, and has conducted over 300 ADI-R evaluations.

Diet. An estimate of dietary intake during the previous week was obtained using Block Brief 2000 Food Frequency Questionnaire (Adult version), from Nutrition Quest (www.nutritionquest.com).

Biological Sample Collection. Fasting whole blood samples were collected in the morning at the Mayo Clinic. Samples were stored at -80^{o}C freezers at Mayo and ASU until all samples were collected, and then all samples were sent together to Metabolon for testing.

Laboratory Tests. Laboratory measurements were conducted by Mayo Clinic, the Metabolic and Oxidative Stress Laboratory at the University of Arkansas for Medical Sciences, and Metabolon Inc. as described below.

Mayo Clinic. Mayo Clinic laboratories measured levels of vitamin B12, folate, methylmalonic acid, homocysteine, isoprostane, vitamin D, vitamin E, hCG, and MTHFR variants as described below.

Vitamin B12 (cyanocobalamin) was measured quantitatively with a Beckman Coulter Access competitive binding immunoenzymatic assay. Briefly, serum is treated with alkaline potassium cyanide and dithiothreitol to denature binding proteins and convert all forms of vitamin B12 to cyanocobalamin. Cyanocobalamin from the serum competes against particle-bound anti-intrinsic factor antibody for binding to intrinsic factor – alkaline phosphatase conjugate. After washing, alkaline phosphatase activity on a chemiluminescent substrate is measured and compared against a multi-point calibration curve of known cyanocobalamin concentrations.

Folate (vitamin B9) was measured quantitatively with a Beckman Coulter Access competitive binding receptor assay. Briefly, serum folate competes against a folic acid – alkaline phosphatase conjugate for binding to solid phase-bound folate binding protein. After washing, alkaline phosphatase activity on a

chemiluminescent substrate is measured and compared against a multi-point calibration curve of known folate concentrations. The Folate assay is designed to have equal affinities for Pteroylglutamic acid (Folic acid) and 5-Methyltetrahydrofolic acid (Methyl-THF), so the result is a measure of both.

Methylmalonic acid was measured quantitatively by liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, serum is mixed with d3-methylmalonic acid as an internal standard, isolated by solid phase extraction, separated on a C18 column, and analyzed in negative ion mode. Chromatographic conditions and mass transitions were chosen to carefully distinguish methylmalonic acid from succinic acid.

Homocysteine was measured quantitatively by LC-MS/MS. Serum is spiked with d8-homocystine as an internal standard, reduced to break disulfide bonds, and deproteinized with formic acid and trifluoroacetic acid in acetonitrile. Measurement of total homocysteine and d4-homocysteine (reduced from d8-homocystine) is performed in positive ion mode with electrospray ionization.

Urine F2-Isoprostane (8-isoprostane) was measured quantitatively by LC-MS/MS after separation from prostaglandin F2 alpha. Urine is spiked with deuterated F2-isoprostane and deuterated prostaglandin F2 alpha, then positive pressure filtered. A mixed mode anion exchange turbulent flow column is used to clean up samples which are then separated on a C8 column and analyzed in negative ion mode.

Vitamin D (25-hydroxyvitamin D2 and D3) was measured quantitatively by LC-MS/MS. D6-25-hydroxyvitamin D3 is added to serum as an internal standard before protein precipitation with acetonitrile. Online turbulent flow chromatography is used to further clean up the samples prior to separation on a C18 column and analysis in positive ion mode. The D2 and D3 forms are measured separately; results are reported as D2, D3, and the sum.

Vitamin E was measured quantitatively by LC-MS/MS. D6-alpha-tocopherol internal standard is added to serum, and proteins are precipitated with acetonitrile. The supernatant is subjected to online turbulent flow for sample cleanup, separated on a C18 column, and analyzed in positive ion mode.

Serum ferritin was measured quantitatively with a Beckman Coulter Access two-site immunoenzymatic (sandwich) assay. Serum ferritin binds mouse anti-ferritin that is immobilized on paramagnetic particles;

ferritin is also bound by a goat anti-ferritin – alkaline phosphatase conjugate. After washing, alkaline phosphatase activity on a chemiluminescent substrate is measured and compared against a multi-point calibration curve of known ferritin concentrations.

MTHFR mutation analysis was performed for the A1298C and C677T variants using Hologic Invader assays. DNA was isolated from whole blood and amplified in the presence of probes for both wildtype and variant sequences. Hybridization of sequence-specific probes to genomic DNA leads to enzymatic cleavage of the probe, releasing an oligonucleotide that binds to a fluorescently labeled cassette. This second hybridization results in generation of a fluorescent signal that is specific to the wildtype or variant allele.

The Metabolic and Oxidative Stress Laboratory (MOSL) located at Arkansas Children's Research Institute performed the measurements described below.

Sample preparation for measurement of plasma methylation and oxidative stress metabolites. For concentration determination of total thiols (homocysteine, cysteine, cyseinyl-glycine, glutamyl-cysteine, and glutathione), the disulfide bonds were reduced and protein-bond thiols were released by the addition of 50 μl freshly prepared 1.43 M sodium borohydride solution containing 1.5 μM EDTA, 66 mM NaOH and 10 μl n-amyl alcohol and added to 200 μl of plasma. After gentle mixing, the solution was incubated at + 4^0C for 30 min with gentle shaking. To precipitate proteins, 250 μl ice cold 10% meta-phosphoric acid was added and the sample was incubated for 20 min on ice. After centrifugation at 18,000 g for 15 min at 4°C, the supernatant was filtered through a 0.2 μm nylon filter and a 20 μl aliquot was injected into the high-performance liquid chromatography (HPLC) system.

For determination of free thiols and methylation metabolites, proteins were precipitated by the addition of 250 μl ice cold 10% meta-phosphoric acid and the sample was incubated for 10 min on ice. Following centrifugation at 18,000 g for 15 min at +4°C, the supernatant was filtered through a 0.2 μm nylon and a 20 μl aliquot was injected into the HPLC system.

HPLC with Coulometric Electrochemical Detection. The methodological details for metabolite elution and electrochemical detection have been described previously (15, 16) The analyses were accomplished

using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C_{18} column (5 μm; 4.6 x 150 mm, MCM, Inc., Tokyo, Japan) obtained from ESA, Inc. (Chemsford, MA). A 20 μl aliquot of plasma extract was directly injected onto the column using Beckman autosampler (model 507E). All plasma metabolites were quantified using a model 5200A Coulochem II electrochemical detector (ESA, Inc., Chelmsford, MA) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). The concentrations of plasma metabolites were calculated from peak areas and standard calibration curves using HPLC software.

Metabolon Inc. Metabolon Inc. conducted measurements of metabolites in whole blood samples in a manner similar to a previous study (17). Briefly, individual samples were subjected to methanol extraction then split into aliquots for analysis by ultrahigh performance liquid chromatography/mass spectrometry (UHPLC/MS). The global biochemical profiling analysis comprised of four unique arms consisting of reverse phase chromatography positive ionization methods optimized for hydrophilic compounds (LC/MS Pos Polar) and hydrophobic compounds (LC/MS Pos Lipid), reverse phase chromatography with negative ionization conditions (LC/MS Neg), as well as a hydrophilic interaction liquid chromatography (HILIC) method coupled to negative (LC/MS Polar) (Evans 2014). All of the methods alternated between full scan MS and data dependent MSn scans. The scan range varied slightly between methods but generally covered 70–1000 m/z.

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards (18).

Statistical Analysis

Univariate Analysis. To conduct a univariate analysis, a test was performed for whether the population means or medians between two populations are equal against the alternative hypothesis that they are not. To determine which testing method to use, the Anderson-Darling test (19) was applied to each sample. If the recorded samples of a particular metabolite or ratio were drawn from two normal

distributions an F-test was subsequently performed to determine whether the population variances of both distributions were identical. If at least one of the two samples of a particular metabolite or ratio was not drawn from a normal distribution, the two-sample Kolmogorov-Smirnov test (20) was applied to examine whether the two samples were drawn from unknown distributions that had the same shape. This pre-analysis yielded four distinct scenarios for a particular metabolite or ratio: (i) both samples were drawn from normal distributions that had identical population variances, (ii) both samples were drawn from a normal distribution with unequal population variances, (iii) both samples were drawn from two unknown distributions that had the same shape and (iv) both samples were drawn from distinctively different distributions. For scenarios (i), (ii), (iii) and (iv) the standard Student t-test (t=), the Welch test (t≠) (21), the Mann-Whitney U test (MW) (22) and the Welch t-test (t≠*) were applied, respectively, for a significance of $\alpha = 0.5$. If a p-value is less than α, we reject the null hypothesis. Conversely, for a p-value above or equal to α, we cannot reject the null hypothesis. For scenario (iv), the result of the hypothesis test was considered undetermined if the *p*-value was close to the significance α, *e.g.* if $p = 0.07$, the hypothesis test is considered undetermined.

Some of the data analyzed below is categorical. In order to analyze this data, a different hypothesis testing method needs to be used than the one described above. The Chi-square test (χ^2) for independence is used in this case. This tests if categorical variables are independent (23). In this case, it would be determining if the recorded categorical variables are dependent on whether or not the mother has previously had a child with ASD.

In order to determine the robustness of the hypothesis tests, the false discovery rates (FDR) for each metabolite were also calculated (24). This was done by calculating the p-values for various combinations of mothers and calculating the fraction of p-values that were considered significant (≤ 0.05) over the total number of p-values. These combinations included every combination leaving one mother out each time, every combination leaving two mothers out at each time, and every combination leaving three mothers out at each time. This led to 1,770 p-values calculated for each metabolite from which the FDR was computed.

The area under the curve (AUC) of the receiver operating characteristic (ROC) curve was also calculated for each metabolite. The ROC curve is a plot of false positive rate (FPR) vs. the true positive rate (TPR). The higher the area under the curve is, the better the measurements are at classifying between the two groups of mothers (25).

A test was considered significant if the p-value was less than or equal to 0.05 and the FDR value was less than or equal to 0.1.

Multivariate Analysis. While the univariate analyses focused on testing for equal population means or medians of individual metabolites/ratios, this does not answer the question of how important the differences in mean or median are to separate the two groups of mothers. In order to examine the extent of the differences within the recorded observations of two samples, Fisher Discriminant Analysis (FDA) was applied (26). This technique defines a projection direction in the data space such that the squared difference between the centers of the projected observations of both samples over the variances of the projected observations is a maximum. The objective function, J, to compute the projection direction is as follows:

$$J = \frac{(\bar{t}_1 - \bar{t}_2)^2}{s_1^2 + s_2^2} \quad \text{(Eq. 1)}$$

Here, $\bar{t}_1 = \frac{1}{n_1}\sum_{i=1}^{n_1} t_{1,i}$ and $\bar{t}_2 = \frac{1}{n_2}\sum_{i=1}^{n_2} t_{2,i}$ are the orthogonally projected means of both samples onto the direction vector and the sample variances of the projected data points are $s_1^2 = \frac{1}{n_1 - 1}\sum_{i=1}^{n_1}\left(t_{1,i} - \bar{t}_1\right)^2$ and $s_2^2 = \frac{1}{n_2 - 1}\sum_{i=1}^{n_2}\left(t_{2,i} - \bar{t}_2\right)^2$. The orthogonal projection of *i*-th observation from the second sample, $\boldsymbol{x}_{2,i}$, is $t_{2,i} = \boldsymbol{x}_{2,i}^T \boldsymbol{p}$, where $\boldsymbol{p}$ is the unit-length direction vector. Note that the projection coordinate, $t_{2,i}$, is often referred to as a score. Essentially, FDA produces a projection direction which represents a tradeoff between optimally separating the two groups of data and minimizing the spread of the projected data within each group. FDA is used to develop a multivariate model that can be used to classify between the two groups of data.

FDA works well with data consisting of real numbers. However, some of the data were discrete in nature such as the information about MTHFR gene mutation. For classification tasks including continuous and

discrete data, logistic regression was used. Logistic regression is similar to linear regression, but the output is variable that can assume two or more discrete values, *i.e*. a binomial or multinomial variable. The prediction of a logistic regression model is the probability that a sample belongs to a particular class. The class that produces the highest probability is considered the class that the model classified the sample as belonging to (27, 28).

The multivariate analysis made use of both FDA and logistic regression. The data was split into multiple subsets for analysis. These subsets include: (i) the 20 measurements from the FOCM/TS pathways, (ii) the same 20 measurements plus additional nutritional information, (iii) the 20 FOCM/TS metabolites with the additional nutritional information and the MTHFR gene information, and (iv) the 20 FOCM/TS metabolites, the additional nutritional information, the MTHFR gene information and a select number of significant metabolites from the broad metabolomics analysis. The 50 metabolites from the Metabolon dataset included in analysis produced highest AUC values from the corresponding ROC curves. This is to reduce the total number of metabolites from 621 to 76 for case (iv). All combinations of two through ten variables were analyzed in each subset. FDA was used for subsets (i) and (ii) and logistic regression was used for subsets (iii) and (iv). This is because subsets (iii) and (iv) contained the MTHFR gene information which are binary variables. The focus on analyzing a reduced set of variables for each of the four cases, instead of just investigating the full variable set, is to examine differences in the FOCM/TS pathways previously found in pregnant women who have had a child with ASD. This is to extend our previous work (8) to apply to women who are not pregnant and to determine what additional information may be important to classify between the two groups of mothers.

In order to determine the most significantly contributing metabolites from the complete set of metabolites, all combinations involving two through ten metabolites were studied. To independently assess the classification accuracy, we used a leave-one-out cross-validatory procedure (LOOCV) (29). LOOCV removes the first observation, determining a model using (Eq. 1) based on the $n-1$ variables, and then applying this model to the first observation which was left out. This application is designed to determine whether this observation is correctly/incorrectly classified as belonging to group 1 or 2. Then, the second observation is left out, whilst the first observation is included for determining a second model using (Eq.

1). The second model is then used to decide whether the second observation is correctly classified or misclassified. Repeating this procedure until each of the observations is left out once allows the calculation of the overall rate of correctly classified and misclassified observations. For determining whether an observation is correctly or incorrectly classified, the samples describing the ASD group were defined as positives and the corresponding samples of the TD cohort as negatives. The decision boundary to assign the label “group 1” or “group 2” to a data point was based on a kernel density estimation of the scores (projection coordinates) computed by the FDA model from the positives (ASD group). More precisely, the decision boundary is determined for a chosen confidence level (one-sided) such that a scores that is less than or equal to this boundary is labeled ASD subject and a score that is larger than this threshold is labeled as a TD subject. The confidence level is chosen in order to reduce the difference between the type I and type II errors.

Results

This section provides information about the study participants, the results of the univariate analysis of the metabolites, the multi-variate analyses for the 4 subsets of metabolites discussed in the methodology section, and lastly a correlation analysis to investigate the grouping of metabolites into five primary groups.

Univariate analysis

Participants. The medical histories and characteristics of the patients are shown in tables 1-3. The hypothesis testing in the tables was done using either Chi-squared test or the Student’s t-test. Table 1 lists basic characteristics of the mothers and their children, the medical histories of the mothers, and the developmental history of their children. Table 2 lists the medications that were being taken by the mothers at the time of the study. Table 3 lists mental and physical symptoms of the mothers. Each table lists n.s. for the p-value or FDR when the result was greater than 0.05 for the p-value or 0.1 for FDR, marking that the measurement showed no statistically significant differences between the two groups. All ASD-M participants enrolled in this study had a child that met full criteria for ASD based on ADI-R scores. The

average age of mothers in the ASD-M group and TD-Group were similar (35.4 years and 34.9 years, respectively), since they were matched for maternal age. The average ages of their children was slightly older for the ASD group (4.71 vs. 3.87 years), as we allowed any ages between 2 and 5 years, and the ASD group was skewed towards the end of that range since it takes time for children with ASD to be diagnosed and for us to have contact with them..

Table 1

Characteristics and medical histories of participants. The means are shown with the standard deviations in parentheses.

The results in Table 1 indicate that besides the child's age, there were no significant differences between the two groups of mothers/children in their medical histories.

Table 2

Current maternal medication use at 2-5 years after pregnancy. The percentage of participants that used these medications is listed with the actual number in parentheses.

	ASD (n=30)	NT (n=29)	Chi-Squared	FDR
Use of Any Medications	43% (13)	30% (9)	n.s	
Psychiatric	17% (5)	7% (2)	n.s.	
Allergy	10% (3)	14% (4)	n.s.	
Birth control	33% (10)	10% (3)	0.03	n.s.
Inhaler	0% (0)	3% (1)	n.s.	
Blood pressure	7% (2)	0% (0)	n.s.	
Thyroid	10% (3)	7% (2)	n.s.	
GI	0% (0)	3% (1)	n.s.	
Pain	0% (0)	3% (1)	n.s.	
Epi-pen as needed	3% (1)	0% (0)	n.s.	
Use of Nutritional Supplements*	7% (2)	7% (2)	n.s.	

**None of these nutritional supplements contained folate or vitamin B12.*

Table 3

Current mental and physical symptoms of mothers. The severity scale was 0=none, 1=mild, 2=moderate, 3=severe. The mean is listed with the standard deviation in parentheses.

	ASD (n=30)	TD (n=29)	Ratio of	t-test	FDR

			ASD/TD		
Fatigue	1.15 (1.0)	0.79 (0.8)	1.46	n.s.	
Fibromyalgia	0.11 (0.6)	0 (0)		n.s.	
Depression	0.67 (0.8)	0.48 (0.7)	1.38	n.s.	
Irritability	0.78 (0.9)	0.71 (0.8)	1.09	n.s.	
Anxiety	1.11 (0.9)	0.75 (1.0)	1.48	0.08	n.s.
Cognition	0.56 (1.0)	0.32 (0.6)	1.73	n.s.	
Attention	0.85 (1.0)	0.32 (0.55)	2.65	0.056	n.s.
Sensory Sensitivity	0.44 (0.7)	0.18 (0.4)	2.49	n.s.	
Stool/GI Problems	0.44 (0.8)	0.71 (1.0)	0.62	n.s.	
Sleep Problems	0.74 (0.9)	0.46 (0.74)	1.60	n.s.	
Headaches	0.56 (0.8)	0.64 (0.7)	0.86	n.s.	
Chemical Sensitivity	0.19 (0.5)	0.14 (0.5)	1.30	n.s.	
Allergies	0.78 (1.0)	0.71 (0.7)	1.09	n.s.	
Expressive Language	0.30 (0.9)	0.11 (0.6)	2.77	n.s.	
Receptive Language	0.31 (0.7)	0.11 (0.4)	2.77	n.s.	
Other symptoms	Multiple sclerosis; very sensitive to alcohol; frequent boils;	Nausea/pain from ovarian cyst; chronic pain; sensitive to loud noises; touch aversive			
Average	0.60 (0.5)	0.44 (0.4)	1.37	n.s.	

The p-values and FDR results in Tables 2 and 3 show that there were no significant differences in the medication use listed and the symptoms experienced between the two groups of mothers during the study period. The slightly higher use of birth control in the ASD group may be due to less desire to having additional children after one child is diagnosed with ASD.

FOCM/TS Metabolites. The univariate results for the FOCM/TS metabolites are shown in Table 4. Levels of vitamin B12 and the SAM/SAH ratio are significantly lower in the ASD-M group compared to the TD-M group, ($p \leq 0.05$, FDR ≤ 0.1). Also, levels of Glu-Cys, fCysteine, and fCystine are significantly higher in the ASD-M group compared to the TD-M group ($p \leq 0.05$, FDR ≤ 0.1).

Table 4

Univariate results for FOCM/TS metabolites and vitamin E, folate, ferritin, B12, MMA, and MTHFR status.

Global metabolic profile- Metabolon. 622 metabolites were measured in whole blood. The univariate analysis for the 50 metabolites from broad metabolomics with the highest AUC values are shown in the Table 5. They are ordered starting with those with the highest AUC. Note that these are semi-quantitative measurements (no absolute values), so only the ratio of ASD-M/TD-M is shown. In almost every case the ASD-M group had lower levels of metabolites than the TD-M group, with the levels of 4-vinylphenol sulfate, NAD+, and three glycine-containing metabolites (gamma-glutamylglycine, cinnamoylglycine, propionylglycine) being especially low (ASD-M/TD-M ratio <0.50). Four metabolites were higher in the ASD-M group (histidylglutamate, asparaginylalanine, dimethyl sulfone, and mannose). Note that dimethyl sulfone was unusually high in the ASD-M group (ASD-M/TD-M ratio = 18.7, p = 0.01, but the FDR was not significant). 80% of the TD-M measurements of dimethyl sulfone and 47% of the ASD-M measurements of dimethyl sulfone were below the detection limit, and the distribution of the data for is skewed.

Table 5

Univariate results of the metabolites from broad metabolomics.

Hypothesis testing was also done on the entire Metabolon dataset and revealed that 48 of these metabolites had significant differences between the two groups of mothers. 3 of these metabolites were not included in the top 50 used for analysis. These were not included in the multivariate analysis because they had lower AUC values than the metabolites included (see Table S-1).

Table 6 contains more information about the metabolites in Table 5. Table 6 lists the many metabolic pathways which had significant differences between the ASD-M and TD-M groups, including amino acids (15 metabolites), carbohydrates (1 metabolite), vitamins (2 metabolites), energy (1 metabolite), lipids (16 metabolites), peptides (4 metabolites), and xenobiotics (7 metabolites). When considering sub-pathways, there were differences in alanine/aspartate metabolism (1 metabolite), glutamate metabolism (3

metabolites), glutathione metabolism (2 metabolites), glycine (1 metabolites), leucine/isoleucine/valine (3 metabolites), polyamine (1 metabolite), tryptophan (2 metabolites), tyrosine (1 metabolite), urea cycle (2 metabolites), fructose/mannose (2 metabolites), nicotinamide (1 metabolite), vitamin B6 (1 metabolite), vitamin B12 (1 metabolite), TCA cycle (1 metabolite), endocannabinoid (1 metabolite), carnitine/fatty acid metabolism (12 metabolites), other fatty acid metabolism (3 metabolites), dipeptides (2 metabolites), gamma-glutamyl (2 metabolites), benzoate (3 metabolites), chemical/xenobiotics (2 metabolites), cinnamoylglycine (1 metabolite), and xanthine metabolism (2 metabolites).

Table 6

Pathways and subpathways of the metabolites from the broad metabolomics.

Carnitine. As shown in Table 6, several carnitine-conjugated metabolites are significantly different in the two groups of mothers. Table 7 below highlights the univariate hypothesis testing results for the carnitine-conjugated metabolites specifically in order of increasing size, from 4-carbon to 24-carbon chains. The ratio of ASD/TD for carnitine-conjugated metabolites was consistently low, ranging from 0.63 to 0.87, with an average of 0.77. There were 33 additional carnitine metabolites in the 600 metabolites measured by untargeted metabolomics. Of these 33, only three had ratios indicating levels of the carnitine were higher in the ASD-M group than in the TD-M group. Also, eight of these metabolites showed significant difference in mean/median between the two groups using hypothesis testing. All of the eight carnitine metabolites had ratios indicating that the levels of carnitine-conjugated molecules in the ASD-M group were less than in the TD-M group.

In contrast, carnitine and two of its precursors (lysine and trimethyllysine) are very similar in the ASD-M and TD-M groups (within 1%), suggesting that the low levels of carnitine-conjugated metabolites is not due to a carnitine deficiency.

Table 7

Univariate hypothesis testing results for the carnitine-conjugated metabolites.

Carnitine	Test	p-Value	FDR	AUC	Ratio (ASD-M/TD-M
Succinylcarnitine (C4-DC)	t=	0.03	0.07	0.68	0.87
Tiglylcarnitine (C5:1-DC)	MW	0.02	0.02	0.67	0.63
Octanoylcarnitine (C8)	MW	4.00E-04	0.00	0.77	0.67
Decanoylcarnitine (C10)	t≠	1.55E-04	0.00	0.78	0.66
Cis-4-decanoylcarnitine (C10:1)	t=	5.82E-04	0.00	0.74	0.75
Laurylcarnitine (C12)	MW	4.30E-03	0.00	0.72	0.74
Myristoylcarnitine (C14)	MW	4.10E-03	0.00	0.72	0.82
Palmitoylcarnitine (C16)	MW	0.02	0.03	0.67	0.83
Arachidoylcarnitine (C20)*	MW	3.00E-03	0.00	0.73	0.85
Eicosenoylcarnitine (C20:1)*	MW	0.02	0.05	0.67	0.83
Arachidonoylcarnitine (C20:4)	MW	0.02	0.00	0.68	0.77
Adrenoylcarnitine (C22:4)*	MW	0.03	0.11	0.67	0.74
Docosapentaenoylcarnitine (C22:5n3)*	MW	0.01	0.00	0.69	0.72
Lignoceroylcarnitine (C24)*	t=	0.02	0.01	0.68	0.84

Note. Statistically significant metabolites with a p-value ≤ 0.05 and FDR ≤ 0.1 are shown in gray.

Multivariate Analysis

The multivariate analysis was performed using multiple subsets of data. The subsets included the twenty metabolites from the FOCM/TS pathways (i), the FOCM/TS metabolites plus some additional nutritional information (ii), the FOCM/TS metabolites plus the additional nutritional information and the MTHFR gene information (iii), and subset (iii) plus fifty metabolites from the broad metabolomics analysis (iv). The first two subsets were analyzed using FDA because all of the variables were continuous and the last two subsets were analyzed using logistic regression because the variables included both continuous and binary data. Each multivariate analysis was combined with leave-one-out cross-validation in order to analyze the success of the model on classification. The best combinations of metabolites from each of the first three subsets had errors ranging from 20-27% which shows only a very modest ability to predict which group of mothers the sample came from. Table 8 below details the type I/type II errors using these metabolites.

Table 8

Results for the combination of metabolites from the first three subsets (i-iii) with lowest errors.

Subset	Combination	Type I Error	Type II Error
(i): FOCM/TS Metabolites	tCysteine, Glu-Cys, fCysteine, fCystine/fCystiene, Nitrotyrosine	24%	27%
(ii): FOCM/TS metabolites plus nutritional information	SAM/SAH, Glu-Cys, GSSG, fCysteine, B12	24%	27%
(iii): FOCM/TS metabolites, nutritional information, and MTHFR gene information	SAM/SAH, tCysteine, Glu-Cys, B12, MTHFR mut. (A1298C)	24%	20%

In order to visually demonstrate the separation between the two groups, a probability density function (PDF) was created for each of the best combinations analyzed using FDA. These PDFs are shown in figures 1 and 2.

Figure 1: PDFs of the combination of metabolites from the FOCM/TS metabolites (i) that resulted in the respective errors shown in table 8.

Figure 2: PDFs of the combination of metabolites from the FOCM/TS metabolites and additional measurements (ii) that resulted in the respective errors shown in table 8.

In order to visually demonstrate the classification accuracy between the two groups when using the logistic regression classification model, a scatter plot was created showing the probabilities of each sample being classified as one group or another. The scatter plot representing the combination of metabolites from the FOCM/TS metabolites plus additional information and the MTHFR gene information (iii) is shown in the figure 3 below.

Figure 3: Scatter plot of the probabilities of being classified into one group or the other using a combination of variables from the FOCM/TS pathways, the additional measurements, and the MTHFR gene information (iii) that resulted in the errors listed in table 8.

The highest accuracies were found when analyzing the fourth, and largest, subset of metabolites. The best combinations for 2, 3, 4, and 5 metabolites for subset iv are shown in Table 9; combinations that contained more than 5 variables resulted in a decrease in accuracy due to overfitting of the classification model. It is important to note that many other combinations of metabolites yielded similar results and the

top combinations of five metabolites are listed in Table 10. The results for using even only two metabolites resulted in lower Type 1 and Type 2 errors than the analysis using the other subsets described above (Table 5) and including more than two metabolites for classification further improved accuracy.

Table 9

Multivariate results using top combinations of 2-5 variables from subset (iv).

Metabolites	Type I Error (FPR)	Type II Error (FNR)
2 metabolites: Histidylglutamate, 6-hydroxyindoel sulfate	17%	13%
3 metabolites: Histidylglutamate, N-formylanthranilic acid, palmitoylcarnitine (C16)	7%	7%
4 metabolites: Histidylglutamate, S-1-pyrroline-5-carboxylate, N-acetyl-2-aminooctanoate*, 5-methylthioadenosine (MTA)	3%	7%
5 Metabolites: Glu-Cys, histidylglutamate, cinnamoylglycine, proline, adrenoylcarnitine (C22:4)*	3%	3%

Table 10

Multivariate results using the top combinations of 5 variables from subset (iv).

Metabolites	Type I Error (FPR)	Type II Error (FNR)
SAM/SAH, percent oxidized, histidylglutamate, cis-4-decenoylcarnitine (C10:1), 3-indoxyl sulfate	3%	7%
fGSH/GSSG, histidylglutamate, 4-vinylphenol sulfate, 3-indroxyl sulfate, palmitoylcarnitine (C16)	3%	7%
Histidylglutamate, 4-vinylphenol sulfate, cinnamoylglycine, N-acetylvaline, palmitoylcarnitine (C16)	3%	7%
Glu-Cys, histidylglutamate, catechol sulfate, phenol sulfate, N-acetyl-2-aminooctanoate*	3%	7%
tGSH, 4-vinylphenol sulfate, 5-oxoproline, asparaginylalanine, tiglylcarnitine (C5:1-DC)	7%	3%

To further illustrate classification accuracy, the 5-metabolite model from Table 9 was used and the probabilities that the samples would be classified by the model in each of the two groups are shown in Figure 1. The metabolites of this 5-metabolite model consisting of Glu-Cys, histidylglutamate, cinnamoylglycine, proline, adrenoylcarnitine (C22:4)* are hereafter referred to as the core metabolites.

Figure 4: Scatter plot of the probabilities of being classified into one group or the other using a combination of variables from the FOCM/TS pathways, the additional measurements, and the top 50 metabolites from the metabolon.

The plots show that the ASD-M samples have a high probability of being classified as ASD-M and the TD-M samples have a high probability of being classified as TD-M. The results from this figure coupled with the low misclassification errors from Table 9 show that there are significant metabolic differences between the two groups of mothers and that these differences are sufficiently large to allow for accurate classification in the vast majority of cases.

In order to further investigate the differences between the two groups, we calculated the correlation coefficients between the 5 metabolites from the best classification model (Table 9) and the rest of the metabolites considered in the analysis for the combined set of ASD-M and TD-M samples. The metabolites that had the highest correlation coefficients with these metabolites are listed in Table 11. We also calculated the correlation of the top 5 metabolites with one another, and, as expected, found very little correlation among these five (see Table 12); this is not unexpected as the classification algorithms tries to identify metabolites that provide new information that can be used for classification as redundant information will not increase classification accuracy. This suggests that there are five general areas of metabolic differences in mothers of children with/without ASD involving 9 or more metabolites for each area.

Table 11

Correlations between the core metabolites and the other 71 analyzed metabolites.

Table 12

Correlation coefficients between the five core metabolite models from Table 9 that provide the highest accuracy.

Metabolites	Correlation Coefficient	P-value
Glu-Cys x Histidylglutamate	-0.01	0.92
Glu-Cys x Cinnamoylglycine	-0.06	0.63
Glu-Cys x Proline	-0.09	0.51
Glu-Cys x Adrenoylcarnitine (C22:4)*	0.01	0.95
Histidylglutamate x Cinnamoylglycine	-0.03	0.81
Histidylglutamate x Proline	-0.07	0.59
Histidylglutamate x Adrenoylcarnitine (C22:4)*	-0.06	0.65
Cinnamoylglycine x Proline	1.80E-03	0.99
Cinnamoylglycine x Adrenoylglycine (C22:4)*	-0.13	0.31
Proline x Adrenoylcarnitine (C22:4)*	0.04	0.74

Most of the metabolites listed in Tables 4 and 5 that were significantly different between the ASD and TD groups were found to be significantly correlated with the 5 core metabolites. However, there were 5 metabolites that were significantly different between the ASD-M and TD-M groups that did not significantly correlate with the 5 core metabolites. These five metabolites were B12, cis-4-decenoylcarnitine (C10:1), catechol sulfate, 7-methylxanthine, and tiglylcarnitine (C5:1-DC). A correlation analysis was conducted to determine if any of the 5 metabolites were correlated with one another, possibly forming a 6th group of correlated metabolites. However, none of the 5 metabolites were significantly correlated with one another. So, it appears that there are 5 primary sets of metabolites, and 5 additional metabolites that are not part of those 5 groups, which are significantly different between the ASD-M and TD-M groups.

Carnitine and Beef

Since the levels of carnitine-conjugated molecules were lower in the ASD-M group (see Table 7), and since beef is the primary dietary source of carnitine (some can also be made by the body), hypothesis testing was performed on the beef quantity and beef frequency in the mother's diets to see if there was a difference between the two groups of mothers. The results are shown below.

Table 13

Univariate hypothesis testing results for beef intake of mothers during pregnancy.

Variable	Test	p-Value	FDR	AUC	Ratio (ASD-M/TD-M)
Beef Frequency	MW	0.73	1.00	0.53	1.12
Beef Quantity	MW	0.83	1.00	0.51	1.41

There was no significant difference found in the mean/median of the beef consumption frequency and quantity between the two groups. Also, the beef consumption frequency and quantity measurements did not significantly correlate with carnitine levels, except for a slight negative correlation of beef frequency and lignoceroylcarnitine (C24) (r= -0.26, p=0.05, unadjusted).

Overall, many of the metabolites measured in this study are significantly different between the two groups of mothers, ASD-M and TD-M. The subset of metabolites that worked the best for classification was a subset of five metabolites which were each correlated with many others.

Discussion

Univariate Analysis

Univariate hypothesis testing was performed to determine if there were significant differences between the two groups of mothers for medical histories and current medications and symptoms. The hypothesis testing done on the medical histories, current medications, and symptoms indicated that there were no significant differences between the two groups other than the age of their children. This shows that the differences that were found in the metabolites were most likely not due to the age of the mothers, their medical histories, current medications, or current symptoms. The first set of univariate hypothesis testing of metabolites involved the metabolites from the FOCM/TS pathways, additional nutritional information, and MTHFR gene information. These hypothesis tests revealed that only five of these measurements have a significant difference in the mean/median between the two groups of mothers (see Table 5).

Hypothesis testing was next performed on the 50 metabolites from Metabolon with the highest AUC. Forty-five of these 50 metabolites were found to have significant differences ($p \leq 0.05$, FDR ≤ 0.1) between the two groups of mothers. Additionally, three other metabolites, not found among the 50 with the highest AUC, also showed statistically significant differences between the two groups (see Table S-1). This reveals that, in addition to the known abnormalities in the FOCM/TS pathway (2-8) there are also many other metabolic pathway differences between mothers of children with/without ASD.

Table 4 lists the significantly different metabolites by pathway, with the primary categories being amino acids, carnitines, and xenobiotics. In almost all cases these particular metabolites were significantly lower in the ASD-M group. This does not appear to be an artifact of the study, because all samples were collected identically and processed and analyzed together, and most metabolites were not significantly different between the ASD-M and TD-M groups. So, the large number of metabolites listed in Tables 6 and 7 suggest that there are in fact many metabolic differences between the ASD-M and TD-M groups.

Multivariate Analysis

FOCM/TS. Multivariate analysis was performed to investigate if the metabolites measured would be able to classify a mother as either having had a child with ASD (ASD-M) or a typically-developing child (TD-M). When using just the metabolites from the FOCM/TS metabolites, a combination of five metabolites appeared to have the lowest misclassification errors calculated using leave-one-out cross-validation. These metabolites included tCysteine, Glu-Cys, fCysteine, fCystine/fCysteine, and Nitrotyrosine. The Type I/Type II errors were approximately 24% and 27%. These errors show that the first subset of metabolites have only modest ability to classify the two groups of mothers.

It is interesting to note that the present results for the FOCM/TS analysis revealed substantially less ability to distinguish the ASD mothers than a similar study (8). The key difference is that the present paper analyzed FOCM/TS metabolites 2-5 years after birth, whereas the other study evaluated mothers during pregnancy; in other words, measurements during pregnancy were better predictors of ASD risk.

FOCM/TS plus nutritional information and MTHFR. The addition of other biomarkers (B12, Folate, Ferritin, MMA, vitamin E, and MTHFR) to the FOCM/TS metabolites did not significantly improve classification with either FDA or logistic regression.

Full set of measurements. The fourth subset of metabolites included the FOCM/TS metabolites, the nutritional biomarkers, the MTHFR gene information, and 50 metabolites from the 600 metabolites measured by Metabolon. Since there were such a large number of measurements from Metabolon, the 50 metabolites with the highest AUC were included in the analysis. This resulted in a total of 77 measurements (50 from the Metabolon data, 20 from FOCM/TS, 5 nutritional biomarkers, and MTHFR information) used for classification. Using this larger set of information, the classification errors decreased significantly. The best combination of five metabolites was found to have misclassification errors as low as 3%. This combination included one metabolite from the FOCM/TS metabolites (Glu-Cys). At least for this study, the metabolites of the FOCM/TS pathway provide some information for a modest classification but other metabolites play an even more important role. Correlation analysis (Table 12) revealed that there appear to be 5 primary categories of significantly different metabolites, with significant correlations within the group to the primary metabolite, but low correlations between the 5 primary metabolites. Almost all of the metabolites which were significantly different between the ASD-M and TD-M groups (see Tables 5 and 6) fell into 1 of these 5 groups. However, there were 5 metabolites that did not significantly correlate with any of the primary metabolites and did not correlate with each other.

Carnitine-conjugated metabolites

The univariate analysis found that all but one carnitine-conjugated metabolite (Adrenoylcarnitine (C22:4)*) were significantly lower in the ASD-M group, with the ratio of carnitine levels for ASD-M/TD-M ranging from 0.66 to 0.87, with an average of 0.78. Carnitine can be produced by the body, but there is some dietary intake also, with the only common dietary sources of carnitine being beef and (to a lesser extent) pork. There were no significant differences in the beef consumption quantity and frequency between the two groups of mothers. Similarly, the levels of carnitine and two of its precursors (lysine and trimethyllysine) were essentially identical for the ASD-M and TD-M groups. This suggests an impairment

in the process of carnitine conjugation, and may be due to a defect or impairment of the enzymes which control carnitine conjugation, such as carnitine palmitoyltransferase 1 (CPT1).

Implication on possible role of nutritional/metabolic status of mothers with children with ASD

Couples who have had a child with ASD have an 18.7% chance of future children being diagnosed with ASD (14), while the general risk for ASD is approximately 1.9% (13). Our results indicate that measurements of Glu-Cys, histidylglutamate, cinnamoylglycine, proline, and adrenoylcarnitine (C22:4)* may be able to predict with approximately 97% accuracy whether a woman, while she is not pregnant, had a child with ASD in the previous 2-5 years. While it is not possible from this study to draw conclusions about nutritional status of the mothers during pregnancy, it is also not possible to rule out that nutritional status differences may have existed.

This research found that several metabolites of the FOCM/TS pathway are abnormal in the ASD-M group, consistent with other studies (2, 7, 8). Furthermore, a meta-analysis of 12 studies (30) found that supplementation with folic acid during pregnancy results in a significantly reduced risk of ASD in the children, with some studies suggesting that folic acid supplementation during the first two months of pregnancy is most important. Levels of folate were not significantly lower in the ASD-M group in this study (17% lower, p=0.20, n.s.), but folate levels were significantly correlated with two of the 5 key metabolites (Glu-Cys and proline). Similarly, vitamin B12 levels were significantly lower in the ASD-M group, and significantly correlated with 6 of the top 50 metabolites, and one study (10) found that abnormal maternal levels of vitamin B12 were associated with an increased risk of ASD, although one small study (31) found no association. Vitamin B12 and folate work together in recycling of homocysteine to methionine, a key step of the FOCM/TS pathway. Based upon these results, it seems possible that appropriate supplementation with vitamin B12 and folate before and/or during pregnancy may help reduce the risk of ASD.

Similarly, the results of this study suggest that low levels of maternal carnitine may be correlated with the likelihood of having had a child later diagnosed with ASD. Previous studies have demonstrated that carnitine supplementation is beneficial for children with ASD (32-34). It is important to note that the results

for this pilot study are for maternal levels post-pregnancy, so they are only suggestive of possible nutritional and metabolic differences during pregnancy.

Limitations of this Study

There were several limitations of the study performed. This is a pilot study with a relatively small sample size. In order to validate these results, further studies with larger sample sizes are needed. Also, the measurements were taken 2-5 years after giving birth the women and therefore, the measurements are only suggestive of differences that might have been present during pregnancy/lactation. However, this study can serve as a guide for future studies done during pregnancy. There was also a discrepancy in the ages of the children in this study. The ages of the mothers were closely matched, but the children the ASD group were slightly older (4.71 +/- 1.0 vs. 3.87 +/- 1.3, p=0.01), indicating that more time since giving birth had passed for the mothers of children with ASD. Future studies should try to match both ages.

Conclusions

In conclusion, this study found many statistically significant differences in metabolites of mothers of children with ASD compared to mothers of typically-developing children, at 2-5 years after birth. A subset of five metabolites was sufficient to differentiate the two groups with approximately 97% accuracy, after leave-one-out cross-validation. Almost all of the metabolites that were significantly different between the two groups were correlated with one of these five metabolites, suggesting that there are at least five areas of metabolic differences between the ASD-M group and the TD-M groups, represented by five metabolites (Glu-Cys, histidylglutamate, cinnamoylglycine, proline, adrenoylcarnitine (C22:4)) which each correlated with many others. The results of this pilot study may be useful for guiding future studies of metabolic risk factors during conception/pregnancy/lactation.

List of Abbreviations

ASD	Autism Spectrum Disorder
TD	Typically-Developing
FOCM	Folate One-Carbon Metabolism
TS	Transsulfuration
ASD-M	Mothers of young children with Autism Spectrum Disorder
TD-M	Mothers of typically-developing young children
AMPSYCAM	ASU-Mayo Pilot Study of Young Children with ASD and their Mothers
ADI-R	Autism Diagnostic Interview-Revised
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
MOSL	The Metabolic and Oxidative Stress Laboratory
HPLC	High-performance liquid chromatography
UHPLC/MS	Ultra-high performance liquid chromatography
LC/MS Pos Polar	Reverse phase chromatography positive ionization method optimized for hydrophilic compounds
LC/MS Pos Lipid	Reverse phase chromatography positive ionization method optimized for hydrophobic compounds
LC/MS Neg	Reverse phase chromatography with negative ionization conditions
HILIC	Hydrophilic interaction liquid chromatography
LC/MS Polar	HILIC method coupled to negative
t=	Student's t-test
t≠	Welch t-test
MW	Mann-Whitney U test
t≠*	Welch t-test without normality criteria being met
χ^2	Chi-squared test
FDR	False discovery rate
AUC	Area under the curve

ROC	Receiver operating characteristic
FPR	False positive rate
TPR	True positive rate
FDA	Fisher Discriminant Analysis
LOOCV	Leave-one-out cross-validation
PDF	Probably density function

Declarations

Ethics Approval and Consent to Participate

All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Consent for Publication

Not applicable

Availability of Data and Materials

The datasets supporting the conclusions of this article are included within the article's additional files.

Competing Interests

JBA, BW, KH, and JH have filed an invention disclosure on the methods described here.

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Author Contributions

JBA wrote most of the proposal and the IRB protocol, and supervised the sample collection. He assisted with data analysis and co-wrote most of the paper with KH.

KH conducted most of the data analysis, and co-wrote most of the paper.

DMC was the study coordinator, consented participants, and collected data and samples.

SJJ oversaw the FOCM/TS measurements.

SBM conducted the FOCM/TS measurements

SR oversaw the Metabolon measurements, wrote the methodology section for those measurements, and helped interpret results.

BKW assisted with the proposal and the IRB protocol.

CLS oversaw routine lab testing and phlebotomy.

ELP conducted the ADI-R evaluations, and oversaw the phlebotomy.

JC assisted with part of the statistical analysis.

JH oversaw the statistical analysis and edited the document.

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Supplemental Material

Metabolites with Significant Differences between ASD-M and TD-M

The full Metabolon dataset contained 595 metabolite measurements. The 50 metabolites with the highest AUC, shown in Table 5, were included in the multivariate analysis. 45 of these metabolites exhibited statistically significant differences in the mean or median between the two groups. There were 3 other metabolites in the full Metabolon dataset that were not included in the multivariate analysis because they had lower AUC values than those in the top 50. These metabolites and their hypothesis testing results are shown in Table S-1.

Table S-1

Metabolites from the Metabolon dataset not included in the top 50 for analysis with significant p-values and FDR values.

Metabolite	Test	p-Value	FDR	AUC
2-hydroxyphenylacetate	t=	0.02	0.02	0.66
N-acetylleucine	MW	0.02	0.02	0.66
Margaroylcarnitine (C17)*	t≠	0.02	0.04	0.65

Note. The p-Values, FDR values, and AUC values are listed in the table and sorted by AUC value (largest to smallest), p-value (smallest to largest), and then FDR value (smallest to largest).

Tables Longer Than Page Length

Table 1

Characteristics and medical histories of participants. The means are shown with the standard deviations in parentheses.

	ASD (n=30)	**TD (n=29)**	**p-Value of t-test (T) or Chi-Squared (C)**	**FDR**
Maternal age	35.4 (5.2)	35.2 (5.8)	n.s. (T)	
Child gender	22 m, 8 f (73% male)	14 m, 14 f (50% male)	0.05 (C)	n.s.
Child age	4.71 (1.0)	3.87 (1.3)	0.0091 (T)	0.00
Child birthweight	7.21 (4.2)	6.20 (4.7)	n.s. (T)	
Pregnancy complications	43% (18% mild, 18% moderate, 7% severe	39% (25% mild, 14% moderate, 0% severe)	n.s. (C)	
Birth complications	50% (36% mild, 11% moderate, 4% severe)	32% (21% mild, 7% moderate, 4% severe)	n.s. (C)	
C-section	43%	29%	n.s. (C)	
Months of Breastfeeding without formula	9.1 (11)	8.4 (9.6)	n.s. (T)	
Months of Breastfeeding with formula	2.9 (4.8)	4.3 (7.1)	n.s. (T)	
Months of formula only	4.4 (5.7)	3.1 (4.2)	n.s. (T)	
Solids introduced	6.4 (1.3)	6.2 (2.6)	n.s. (T)	
Prenatal usage	89%	93%	n.s. (C)	
% used prenatal prior to conception	37%	37%		
Week started prenatal (with preconception use scored as week zero)	2.8 (3)	3.8 (4)	n.s. (T)	
Pesticide exposure	14%	11%	n.s. (C)	
% organic food	24% (26%)	24% (25%)	n.s. (T)	
Child's Antibiotic usage (Rounds, where 1 round=10 days)				
0-6 months	0.22 (0.6)	0.29 (0.5)	n.s. (T)	
6-12 months	0.73 (1.3)	0.79 (1.0)	n.s. (T)	
12-24 months	1.08 (2.0)	1.25 (1.7)	n.s. (T)	
24-36 months	1.12 (1.1)	0.74 (1.1)	n.s. (T)	
36-48 months	0.77 (1.1)	0.36 (0.6)	n.s. (T)	
Total antibiotic usage 0-48 months	3.29 (4.1)	3.24 (3.0)	n.s. (T)	
Child's Asthma severity	3% 1 mild	18% 3 mild, 1 moderate, 1 severe	n.s. (C)	
Child's Food allergies/sensitivities (severity based on	32% (7% mild 14% moderate	11% (4% mild, 7% moderate 0% severe)	n.s. (C)	

most severe allergy)	11% severe)		
Other allergies (severity based on most severe allergy)	14% (7% mild, 4% moderate, 4% unknown)	33% (19% mild, 15% moderate, 0% severe)	n.s. (C)
Child's Developmental history of ASD			
Early Onset	14%		
Normal development, then regression (age of regression)	46% 19 (5) months		
Normal development, then plateau (age of plateau)	39% 19 (9) months		

Table 4

Univariate results for FOCM/TS metabolites and vitamin E, folate, ferritin, B12, MMA, and MTHFR status.

Metabolite	**Test**	**ASD-M (mean ± std) N= 30**	**TD-M (mean ± std) N= 29**	**p-Values**	**FDR**	**AUC**	**Ratio (ASD-M/TD-M)**
B12Δ	MW	355 ± 196	473 ± 173	2.40E-03	0.00	0.73	0.75
fCysteine Δ	t=	23.8 ± 1.88	22.6 ± 1.94	0.01	0.00	0.70	1.06
Glu-Cys Δ	t=	1.89 ± 0.22	1.72 ± 0.24	0.01	0.00	0.69	1.10
SAM/SAH Δ	t=	1.94 ± 0.25	2.09 ± 0.20	0.01	0.00	0.67	0.93
fCystine Δ	t=	24.1 ± 2.78	22.4 ± 2.43	0.02	0.01	0.67	1.07
tCysteine	t=	248 ± 23.1	234 ± 28.3	0.04	0.40	0.64	1.06
tGSH	t=	6.23 ± 0.98	5.85 ± 1.07	0.15	1.00	0.63	1.07
SAM	t=	47.2 ± 5.37	49.3 ± 5.76	0.15	1.00	0.62	0.96
Methionine	t=	19.9 ± 2.56	20.8 ± 2.98	0.19	1.00	0.61	0.95
MTHFR mut. (A1298C)	χ^2			0.15	1.00	0.60	
tGSH/GSSG	t=	29.5 ± 6.21	27.3 ± 6.06	0.18	1.00	0.60	1.08
Folate	MW	17.6 ± 6.19	21.1 ± 9.17	0.20	1.00	0.60	0.83
Homocysteine	MW	8.63 ± 0.98	8.27 ± 1.18	0.21	1.00	0.60	1.04
tGSH/GSSG	t=	29.5 ± 6.21	27.3 ± 6.06	0.18	1.00	0.60	1.08
SAH	t=	24.5 ± 2.66	23.6 ± 2.04	0.15	1.00	0.59	1.04
Vitamin D3*	t=	27.1 ± 9.10	24.9 ± 6.08	0.27	1.00	0.57	1.09
Ferritin	MW	35.1 ± 31.0	29.5 ± 26.1	0.36	1.00	0.57	1.19
Cys-Gly	t=	38.7 ± 5.06	37.6 ± 6.38	0.46	1.00	0.57	1.03
Adenosine	t=	0.22 ± 0.03	0.21 ± 0.03	0.28	1.00	0.55	1.04
fGSH/GSSG	MW	8.73 ± 2.08	8.81 ± 1.84	0.49	1.00	0.55	0.99
% Oxidized Glutathione	MW	0.19 ± 0.03	0.19 ± 0.04	0.49	1.00	0.55	1.01
Chlorotyrosine	t=	26.8 ± 4.27	27.6 ± 4.23	0.51	1.00	0.55	0.97
Nitrotyrosine	t≠	32.9 ±	33.7 ± 4.67	0.59	1.00	0.55	0.98

		6.28					
fGSH	t=	1.85 ± 0.32	1.89 ± 0.35	0.62	1.00	0.55	0.98
fCystine/fCysteine	t=	1.01 ± 0.11	1.00 ± 0.10	0.59	1.00	0.54	1.02
Vitamin E	t=	9.23 ± 2.53	9.82 ± 3.22	0.75	1.00	0.54	0.94
Isoprostane (U)*	MW	0.15 ± 0.10	0.18 ± 0.14	0.70	1.00	0.53	0.83
MTHFR mut. (C677T)	χ^2			0.90	1.00	0.53	
GSSG	t=	0.22 ± 0.04	0.22 ± 0.03	0.93	1.00	0.52	1.00
MMA	MW	0.15 ± 0.06	0.15 ± 0.08	0.64	1.00	0.51	0.96

*Note. The measurements are ordered by decreasing AUC. Statistically significant metabolites with p-value ≤ 0.05 and FDR ≤ 0.1 are marked with Δ and * indicates measurements that were left out of the classification procedure as the measurements were not collected from all mothers. Specifically, these were vitamin D with 28 mothers in ASD-M and 28 TD-M mothers and Isoprostane with 28 participants that were ASD-M and 25 mothers in TD-M.*

Table 5

Univariate results of the metabolites from broad metabolomics.

Metabolite	Test	p-Value	FDR	AUC	Ratio (ASD-M/TD-M)
Fructose Δ	t≠*	6.88E-04	0.00	0.81	0.60
Histidylglutamate Δ	t≠	2.67E-05	0.00	0.80	1.50
Decanoylcarnitine (C10) Δ	t≠	1.55E-04	0.00	0.78	0.66
S-1-pyrroline-5-carboxylate Δ	MW	3.09E-04	0.00	0.77	0.63
Octanoylcarnitine (C8) Δ	MW	4.00E-04	0.00	0.77	0.67
4-vinylphenol sulfate Δ	t≠*	1.30E-03	0.00	0.77	0.31
Cis-4-decenoylcarnitine (C10:1) Δ	t=	5.82E-04	0.00	0.74	0.75
N-formylanthranilic acid Δ	t=	1.20E-03	0.00	0.74	0.69
N-acetylasparagine Δ	t≠*	1.90E-03	0.00	0.73	0.78
Arachidoylcarnitine (C20)* Δ	MW	3.00E-03	0.00	0.73	0.85
N-palmitoylglycine Δ	t=	2.20E-03	0.00	0.72	0.81
Citrulline Δ	t=	2.60E-03	0.00	0.72	0.91
6-hydroxyindole sulfate Δ	t≠	1.20E-03	0.00	0.72	0.59
N-palmitoylserine Δ	MW	4.00E-03	0.00	0.72	0.77
Myristoylcarnitine (C14) Δ	MW	4.10E-03	0.00	0.72	0.82
Laurylcarnitine (C12) Δ	MW	4.30E-03	0.00	0.72	0.74
Stearoylcarnitine (C18) Δ	MW	0.01	0.00	0.71	0.85
Gamma-glutamylglycine Δ	t≠*	2.20E-03	0.00	0.71	0.27
5-oxoproline Δ	t=	0.01	0.00	0.70	0.94
Asparaginylalanine Δ	t=	3.90E-03	0.00	0.70	1.32
Glutamine Δ	t=	0.02	0.00	0.70	0.95
Catechol sulfate Δ	MW	0.01	0.00	0.70	0.67
3-indoxyl sulfate Δ	t≠	2.70E-03	0.00	0.70	0.67
7-methylxanthine Δ	MW	0.01	0.00	0.70	0.58
Phenol sulfate Δ	MW	0.01	0.00	0.70	0.67
Cinnamoylglycine Δ	t≠*	0.01	0.00	0.70	0.46
Alpha-ketoglutaramate* Δ	t=	0.02	0.00	0.70	0.73
Isovalerylglycine Δ	MW	0.01	0.00	0.69	0.78
Propionylglycine Δ	MW	0.01	0.00	0.69	0.48
Docosapentaenoylcarnitine (C22:5n3)* Δ	MW	0.01	0.00	0.69	0.72
N-acetyl-2-aminooctanoate* Δ	t≠	3.20E-03	0.00	0.69	0.54
S-methylglutathione Δ	t=	0.02	0.01	0.69	0.86
Gamma-glutamyltyrosine Δ	MW	0.02	0.00	0.68	0.64
Succinylcarnitine (C4-DC) Δ	t=	0.03	0.07	0.68	0.87
Arachidonoylcarnitine (C20:4) Δ	MW	0.02	0.00	0.68	0.77
Glycine Δ	t=	0.01	0.00	0.68	0.87
N-acetylvaline	t=	0.04	0.28	0.68	0.80
Lignoceroylcarnitine (C24)* Δ	t=	0.02	0.01	0.68	0.84
Guaiacol sulfate Δ	MW	0.02	0.01	0.68	0.81
5-methylthioadenosine (MTA) Δ	MW	0.02	0.00	0.68	0.86
Proline Δ	MW	0.02	0.00	0.68	0.90
Pyridoxate Δ	MW	0.02	0.00	0.68	0.75
Palmitoylcarnitine (C16) Δ	MW	0.02	0.03	0.67	0.83
Eicosenoylcarnitine (C20:1)* Δ	MW	0.02	0.05	0.67	0.83
Nicotinamide adenine dinucleotide (NAD+)	t≠*	0.03	0.05	0.67	0.41

Δ					
Dimethyl sulfone	MW	0.01	0.23	0.67	18.7
Tiglylcarnitine (C5:1-DC) Δ	MW	0.02	0.02	0.67	0.63
Adrenoylcarnitine (C22:4)*	MW	0.03	0.11	0.67	0.74
3-methylxanthine	MW	0.03	0.13	0.67	0.74
Mannose	MW	0.03	0.19	0.67	1.21

Note. The metabolites listed here are the 50 metabolites measured by Metabolon from broad metabolomics with the highest area under the receiver operating characteristic (ROC) curve (AUC). Metabolites with p-value ≤ 0.05 and FDR≤0.1 marked with **Δ**.

Table 6

Pathways and subpathways of the metabolites from the broad metabolomics.

Metabolite	Pathway	Sub-Pathway	Higher/lower in ASD-M group
N-acetylasparagine Δ	Amino Acid	Alanine and Aspartate Metabolism	↓
S-1-pyrroline-5-carboxylate Δ	Amino Acid	Glutamate Metabolism	↓
Glutamine Δ	Amino Acid	Glutamate Metabolism	↓
Alpha-ketoglutaramate* Δ	Amino Acid	Glutamate Metabolism	↓
5-oxoproline Δ	Amino Acid	Glutathione Metabolism	↓
S-methylglutathione Δ	Amino Acid	Glutathione Metabolism	↓
Glycine	Amino Acid	Glycine, Serine and Threonine Metabolism	↓
Isovalerylglycine Δ	Amino Acid	Leucine, Isoleucine and Valine Metabolism	↓
N-acetylvaline Δ	Amino Acid	Leucine, Isoleucine and Valine Metabolism	↓
Tiglylcarnitine (C5:1-DC) Δ	Amino Acid	Leucine, Isoleucine and Valine Metabolism	↓
5-methylthioadenosine (MTA) Δ	Amino Acid	Polyamine Metabolism	↓
N-formylanthranilic acid Δ	Amino Acid	Tryptophan Metabolism	↓
3-indoxyl sulfate Δ	Amino Acid	Tryptophan Metabolism	↓
Phenol sulfate Δ	Amino Acid	Tyrosine Metabolism	↓
Citrulline Δ	Amino Acid	Urea cycle; Arginine and Proline Metabolism	↓
Proline Δ	Amino Acid	Urea cycle; Arginine Proline Metabolism	↓
Mannose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	↑
Fructose Δ	Carbohydrate	Fructose, Mannose, and Galactose Metabolism	↓
Nicotinamide adenine dinucleotide (NAD+) Δ	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	↓
Pyridoxate Δ	Cofactors and Vitamins	Vitamin B6 Metabolism	↓
Succinylcarnitine (C4-DC) Δ	Energy	TCA Cycle	↓
N-palmitoylserine Δ	Lipid	Endocannabinoid	↓
Decanoylcarnitine (C10) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Octanoylcarnitine (C8) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Cis-4-decenoylcarnitine (C10:1) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Arachidoylcarnitine (C20)* Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Myristoylcarnitine (C14) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Laurylcarnitine (C12) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓

Stearoylcarnitine (C18) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Docosapentaenoylcarnitine (C22:5n3)* Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Arachidonoylcarnitine (C20:4) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Lignoceroylcarnitine (C24)* Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Palmitoylcarnitine (C16) Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Eicosenoylcarnitine (C20:1)* Δ	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
Adrenoylcarnitine (C22:4)*	Lipid	Fatty Acid Metabolism (Acyl Carnitine)	↓
N-palmitoylglycine Δ	Lipid	Fatty Acid Metabolism (Acyl Glycine)	↓
Propionylglycine Δ	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	↓
N-acetyl-2-aminooctanoate* Δ	Lipid	Fatty Acid, Amino	↓
Histidylglutamate Δ	Peptide	Dipeptide	↑
Asparaginylalanine Δ	Peptide	Dipeptide	↑
Gamma-glutamylglycine Δ	Peptide	Gamma-glutamyl Amino Acid	↓
Gamma-glutamyltyrosine Δ	Peptide	Gamma-glutamyl Amino Acid	↓
4-vinylphenol sulfate Δ	Xenobiotics	Benzoate Metabolism	↓
Catechol sulfate Δ	Xenobiotics	Benzoate Metabolism	↓
Guaiacol sulfate Δ	Xenobiotics	Benzoate Metabolism	↓
6-hydroxyindole sulfate Δ	Xenobiotics	Chemical	↓
Dimethyl sulfone	Xenobiotics	Chemical	↑
Cinnamoylglycine Δ	Xenobiotics	Food Component/Plant	↓
7-methylxanthine Δ	Xenobiotics	Xanthine Metabolism	↓
3-methylxanthine	Xenobiotics	Xanthine Metabolism	↓

Note. The metabolites listed here are the 50 metabolites measured by Metabolon from broad metabolomics with the highest area under the receiver operating characteristic (ROC) curve (AUC). The metabolites are sorted alphabetically by pathway and then subpathway. A fourth column lists whether the metabolites were higher or lower in the ASD-M group. Metabolites that had a p-value ≤0.05 and FDR ≤ 0.1 (FDR-values listed in Table 3) are marked by Δ.

Table 11

Correlations between the core metabolites and the other 71 analyzed metabolites.

Metabolite	Correlation Coefficient	p-Value
Glu-Cys		
tGSH	0.55	5.71E-06
tGSH/GSSG	0.35	0.01
6-hydroxyindole sulfate	-0.25	0.05
SAM/SAH	-0.26	0.04
N-formylanthranilic acid	-0.28	0.03
5-methylthioadenosine (MTA)	-0.28	0.03
Pyridoxate	-0.31	0.02
Folate	-0.38	3.40E-03
Histidylglutamate		
Asparaginylalanine	0.55	6.74E-06
Mannose	0.40	1.70E-03
fCystine	0.30	0.02
Succinylcarntine (C4-DC)	-0.27	0.04
Citrulline	-0.27	0.04
Fructose	-0.28	0.03
Octanoylcarnitine (C8)	-0.29	0.02
Gamma-glutamylglycine	-0.30	0.02
Isovaleryglycine	-0.32	0.01
Decanoylcarnitine (C10)	-0.33	0.01
Cinnamoylglycine		
N-acetyl-2-aminooctanoate*	0.45	4.13E-04
N-formylanthranilic acid	0.44	4.30E-04
3-indoxyl sulfate	0.35	0.01
Citrulline	0.33	0.01
6-hydroxyindole sulfate	0.32	0.01
Chlorotyrosine	0.29	0.02
Alpha-ketoglutaramate*	0.28	0.03
Nicotinamide adenine dinucleotide (NAD+)	0.28	0.03
Pyridoxate	0.27	0.04
Guaiacol sulfate	0.26	0.04
S-methylglutathione	0.26	0.04
Methionine	-0.29	0.02
fCysteine	-0.33	0.01
Proline		
S-1-pyrroline-5-carboxylate	0.59	1.22E-06
Gamma-glutamyltyrosine	0.45	3.73E-04
3-indoxyl sulfate	0.44	5.33E-04
6-hydroxyindole sulfate	0.43	6.01E-04
Phenol sulfate	0.41	1.20E-03
Glutamine	0.36	0.01
Propionylglycine	0.35	0.01
Glycine	0.35	0.01
Gamma-glutamylglycine	0.32	0.01
5-oxoproline	0.30	0.02
Alpha-ketoglutaramate*	0.28	0.03
Folate	0.28	0.03
N-formylanthranilic acid	0.27	0.04

Adenosine	-0.30	0.02
Adrenoylcarnitine (C22:4)*		
Arachidonoylcarnitine (C20:4)	0.93	8.00E-26
Docosapentaenoylcarnitine (C22:5n3)*	0.85	8.64E-18
Eicosenoylcarnitine (C20:1)*	0.74	2.35E-11
Palmitoylcarnitine (C16)	0.70	8.13E-10
Myristoylcarnitine (C14)	0.69	2.17E-09
Laurylcarnitine (C12)	0.49	8.88E-05
Fructose	0.41	1.20E-03
N-acetylasparagine	0.38	2.60E-03
Stearoylcarnitine (C18)	0.31	0.02
Methionine	0.26	0.04
Cys-Gly	0.26	0.05
Arachidoylcarnitine (C20)*	0.26	0.05
N-palmitoylserine	0.25	0.05
fCysteine/fCystine	-0.29	0.03
fCystine	-0.30	0.02