UC Davis
UC Davis Previously Published Works

Title

Maternal metabolic profile predicts high or low risk of an autism pregnancy outcome

Permalink

https://escholarship.org/uc/item/6xd69984

Authors

Hollowood, Kathryn
Melnyk, Stepan
Pavliv, Oleksandra
et al.

Publication Date

2018-12-01

DOI

10.1016/j.rasd.2018.09.003

Peer reviewed

Maternal metabolic profile predicts high or low risk of an autism pregnancy outcome

Kathryn Hollowood[a,b], **Stepan Melnyk**[c], **Oleksandra Pavliv**[c], **Teresa Evans**[c], **Ashley Sides**[d], **Rebecca J. Schmidt**[e], **Irva Hertz-Picciotto**[e], **William Elms**[e], **Elizabeth Guerrero**[e], **Uwe Kruger**[a], **Juergen Hahn**[a,b,f], and **S. Jill James**[c]

[a]Department of Biomedical Engineering, Rensselaer Polytechnic Institute, 110 8th St, Troy, NY, 12180, USA

[b]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th St, Troy, NY, 12180, USA

[c]Department of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Research Institute, 4301 W Markham St, Little Rock, AR, 72205, USA

[d]Translational Research Institute, University of Arkansas for Medical Sciences, 1301 W Markham St, Little Rock, AR, 72205, USA

[e]Department of Public Health Sciences and the MIND Institute, University of California Davis School of Medicine, 1 Shields Ave, Davis, CA, 95616, USA

Corresponding author: Juergen Hahn, PhD, Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, 110 8th Street, Troy, NY 12180-3590, hahnj@rpi.edu.
Author Note
The authors are: Kathryn Hollowood, Department of Biomedical Engineering and Center for Biotechnology & Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA; Stepan Melnyk, Department of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Research Institute, Little Rock, AR, USA; Oleksandra Pavliv, Department of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Research Institute, Little Rock, AR, USA; Teresa Evans, Department of Pediatrics, University of Arkansas for Medical Sciences, Arkansas Children's Research Institute, Little Rock, AR, USA; Ashley Sides, Translational Research Institute, University of Arkansas for Medical Sciences, Little Rock, AR, USA; Rebecca J. Schmidt, Department of Public Health Sciences and the MIND Institute, University of California Davis School of Medicine, Davis, CA, USA; Irva Hertz-Picciotto, Department of Public Health Sciences and the MIND Institute, University of California Davis School of Medicine, Davis, CA, USA; William Elms, Department of Public Health Sciences and the MIND Institute, University of California Davis School of Medicine, Davis, CA, USA; Elizabeth Guerrero, Department of Public Health Sciences and the MIND Institute, University of California Davis School of Medicine, Davis, CA, USA; Uwe Kruger, Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA; Juergen Hahn, Department of Biomedical Engineering and Department of Chemical & Biological Engineering and Center for Biotechnology & Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA; S. Jill James, Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR, USA.

Conflict of Interest
The authors declare that they have no conflict of interest.

Compliance with Ethical Standards
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.

There have been no changes to author affiliation subsequent to the time of the study.

[f]Department of Chemical & Biological Engineering, Rensselaer Polytechnic Institute, 110 8th St, Troy, NY, 12180, USA

Abstract

Background: Currently there is no test for pregnant mothers that can predict the probability of having a child that will be diagnosed with autism spectrum disorder (ASD). Recent estimates indicate that if a mother has previously had a child with ASD, the risk of having a second child with ASD is ~18.7% (High Risk) whereas the risk of ASD in the general population is ~1.7% (Low Risk).

Methods: In this study, metabolites of the folate-dependent transmethylation and transsulfuration biochemical pathways of pregnant mothers were measured to determine whether or not the risk of having a child with autism could be predicted by her metabolic profile. Pregnant mothers who have had a child with autism before were separated into two groups based on the diagnosis of their child whether the child had autism (ASD) or not (TD). Then these mothers were compared to a group of control mothers who have not had a child with autism before. A total of 107 mothers were in the High Risk category and 25 mothers in the Low Risk category. The High Risk category was further separated into 29 mothers in the ASD group and 78 mothers in the TD group.

Results: The metabolic results indicated that among High Risk mothers, it was not possible to predict an autism pregnancy outcome. However, the metabolic profile was able to predict with approximately 90% sensitivity and specificity whether a mother fell into the High Risk group (18.7% risk) or Low Risk group (1.7% risk).

Conclusions: Based upon these measurements it is not possible to determine during a pregnancy if a child will be diagnosed with ASD by age 3. However, differences in the folate-dependent transmethylation and transsulfuration metabolites are indicative of the risk level (High Risk of 18.7% vs. Low Risk of 1.7%) of the mother for having a child with ASD.

Keywords

Introduction

Progress in early diagnosis and treatment of autism spectrum disorder has been hindered by the lack of understanding of the underlying pathogenesis of the disorder which is a necessary prerequisite for the design of effective treatment and prevention strategies. Although gene-environment interactions are thought to be involved, as of yet none have been reproducibly identified. The metabolic basis for autism has received much less research attention despite the fact that chronic biochemical imbalance is often a primary factor in the development of complex diseases and has been implicated in the pathogenesis of multiple other neurobehavioral disorders (Andreazza et al., 2009; Dean et al., 2009; Gysin et al., 2007; Mattson & Shea, 2003; Smythies, Gottfries, & Regland, 1997). A targeted candidate pathway approach to autism pathogenesis offers advantages over untargeted genomic/

proteomic approaches by providing a reflection of the combined influence of genes and environment on a defined metabolic phenotype.

In this study, we target the tetrahydrofolate (THF)-dependent methionine transmethylation and transsulfuration (TM/TS) pathways for glutathione (GSH) synthesis in pregnant mothers at high risk of having a second child with autism (Figure 1). The vital importance of these three interconnected pathways during gestation is underscored by their essentiality for error-free DNA synthesis and repair, cell proliferation, and immune potential (Pathway 1); for essential cellular methylation reactions including DNA, RNA, proteins, phospholipids, and neurotransmitters (Pathway 2); and for the maintenance of glutathione (GSH) redox homeostasis for cell signaling, detoxification, stress response, cell cycle progression and apoptosis (Pathway 3). Because these three pathways regulate the distribution of precursors for DNA synthesis (proliferation), DNA/histone methylation (epigenetics) and glutathione synthesis (antioxidant/detoxification potential), the homeostatic balance between these pathways is essential to support normal cell programming and ontogeny during prenatal and post-natal development (Chmurzynska, 2010; Rassin, Sturman, & Gaull, 1981; Zeisel, 2009). Viewed in the context of systems biology, these are clearly core metabolic pathways that represent "hubs" for the regulation of gene expression and redox signaling during rapid fire shifts between proliferation, differentiation and cell death during fetal development. The biochemical details of these pathways details are included in the Figure 1 legend.

Alterations in the prenatal intrauterine metabolic environment can have a profound influence on fetal brain development and have been shown to confer risk or resistance to subsequent brain and behavioral dysfunction (Bassan et al., 2010; Brown et al., 2007; Lowery, Govindan, Murphy, & Eswaran, 2008; Rees, Harding, & Walker, 2008; Takao & Miyakawa, 2009; Yuan, Sun, Zhan, & Yu, 2010). Normal fetal neurodevelopment depends on precise temporal and spatial pattern of gene expression that is predominantly regulated by epigenetic mechanisms and redox signaling of neural precursor cells to differentiate, proliferate or die. Thus, an imbalance in metabolic precursors for epigenetic regulation or redox signal transduction during critical developmental windows can misdirect precursor cell fate leading to downstream derangement of brain cell ontogeny and neurologic function relevant to autism pathogenesis (Beaudin & Stover, 2009; Chmurzynska, 2010; Furness, Fenech, Khong, Romero, & Dekker, 2008; Rassin et al., 1981; Taparia, Gelineau-van Waes, Rosenquist, & Finnell, 2007; Wells et al., 2009).

The estimated risk of having a child with autism is approximately 1.7% in the general population (Baio, 2018) whereas the risk of having autism among mothers who have previously had a child with autism is estimated to be ~18.7% (Ozonoff et al., 2011). Currently there is no test for pregnant mothers that can predict the probability of having a child that will be diagnosed with ASD. Previously, we compared folate-dependent TM/TS metabolites in mothers of children with autism 3-10 years after birth and found significant imbalance in several metabolites relative to Low Risk mothers (James et al., 2008). More recently, our research group was able to predict the autism diagnosis with high accuracy in 3-10 year old children based on multivariate analysis of their TM/TS metabolites (Howsmon, Kruger, Melnyk, James, & Hahn, 2017) as well as for 2-17 year old children from a separate study (Howsmon et al., 2018). Here we extend this work to pregnant

mothers to determine whether TM/TS metabolites can be used as biomarkers to predict (1) the risk of whether a High Risk mother will have a child diagnosed with ASD at age 3 and (2) whether TM/TS metabolites can be used to predict whether the overall maternal risk of having a child with ASD falls into the ~1.7% (Low Risk) or the ~18.7% (High Risk) category.

Materials and Methods

High Risk Pregnant Mothers

A total of 146 mothers were recruited as part of the MARBLES (Markers of Autism Risk in Babies – Learning Early Signs) Study, a prospective study that enlists mothers of children with ASD who are in a subsequent pregnancy and therefore at a higher risk for delivering a child who develops ASD (Hertz-Picciotto et al., 2018). The MARBLES families were identified from lists of children diagnosed with autism who were receiving services through the California Department of Developmental Services, from other studies at the Medical Investigations of Neurodevelopmental Disorders (MIND) Institute, and by self-referral. Inclusion criteria included: 1) maternal age 18 years; 2) currently pregnant with one or both parents being the biological parent of a previous child with ASD; and 3) residence within the specified area in California. The diagnosis of autism in the younger sibling was obtained by trained administrators at age 36 months using standard ADOS and Mullen Scales of Early Learning diagnostic tools. The Institutional Review Board of the University of California at Davis and the State of California Committee for the Protection of Human Subjects approved the study, and informed consent was obtained at enrollment.

There were a total of 146 mothers in the High Risk group which included three subgroups: 29 of them delivered children with an ASD diagnosis at 36 months, 39 of the children were diagnosed with a developmental delay (DD), and 78 were typically developing. The corresponding percentages are 19.9% ASD, 26.7% DD, and 53.4% TD. As the DD Dx may not be stable (Elmose et al., 2014; Hedvall et al., 2014; Keogh, Bernheimer, & Guthrie, 1997; Roberts, Anderson, Doyle, & Group, 2010) data from this subgroup were not further analyzed in this work which reduces the sample size to 107 mothers.

Low Risk Pregnant Mothers

Recruitment of 25 pregnant Low Risk mothers was done in collaboration with the OB/GYN Department and the Translational Research Institute at UAMS. All mothers lived within a 50 mile radius of Little Rock Arkansas. Inclusion factors included age 18 years with no family history of autism, neurologic or genetic disorders, autoimmune disease, developmental delay, fragile X, or Rett syndrome. The number of Low Risk participants was limited by funding availability. The protocol was approved by the Institutional Review Board at UAMS and all participants signed informed consent before blood draw.

A demographic comparison of mothers in the High Risk mothers (ASD and TD outcomes) compared to the Low Risk mothers demographics is shown in Table 1 below.

Blood Samples

Blood samples were obtained from the mothers at three different time points: 1st trimester, 2nd trimester, and 3rd trimester. The data for the mothers with High Risk were separated as either autism spectrum disorder (ASD), typically developing (TD), or developmental delay (DD) based on the child's follow-up diagnosis at age three.

A summary of the number of participants for each group during each of the three trimesters is given in Table 2 below.

Metabolite Analysis

Blood samples were collected into sodium citrate vacutainer tubes, chilled before centrifugation at $4000 \times g$ and aliquots of plasma were transferred into cryostat tubes and stored at $-80°C$. All samples were collected and frozen between 2006 and 2014 and never thawed before analysis. Plasma samples from California were sent by FEDEX Express on dry ice to the James' laboratory at the Arkansas Children's Research Institute for HPLC analysis. Metabolites in the folate-dependent transmethylation and transsulfuration pathways were measured blinded to technicians. Metabolites of the transmethylation pathway included methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), adenosine, and homocysteine. Metabolites measured in the transsulfuration pathway included total cysteine (tCysteine), free reduced cysteine (fCysteine), free oxidized cysteine (fCystine), glutamyl cysteine (Glu-Cys), cysteinylglycine (Cys-Gly), total glutathione (tGSH), free glutathione (fGSH), and free oxidized glutathione disulfide (GSSG). Measures of oxidative stress included the oxidized tyrosine residues, 3-nitrotyrosine (NT) and 3-chlorotyrosine (C1T) as well as the redox ratios tGSH/GSSG, fGSH/GSSG and cysteine/cysteine. Details of the HPLC detection with electrochemical detection and analysis with internal standards have been previously described (Melnyk et al., 2012).

Statistical Methods

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 (Anderson & Darling, 1954) was applied to each sample. If the recorded samples of a particular metabolite or ratio were drawn from two normal distributions a 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 (Massey, 1951) 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, the Welch test (Welch, 1947), the Mann-Whitney U test (Mann & Whitney, 1947) and the Welch t-test were applied, respectively. For scenario (iv), the result of the hypothesis test was

declared as undetermined if the *p*-value was close to the significance α, which is 0.05 for each test applied, e.g. if $p = 0.07$, the hypothesis test would be declared as undetermined.

In order to show the robustness of the hypothesis tests, the false discovery rates (FDR) for each metabolite were also calculated (Storey, 2002). 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 a time, every combination leaving two mothers out at a time, and every combination leaving three mothers out at a time. This led to several thousand p-values calculated for each metabolite from which the FDR was computed.

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 focus on testing for equal population means or medians of individual metabolites/ratios, this does not answer the question of how significant the differences in mean or median are. In order to examine the extent of the differences within the recorded observations of two samples, Fisher Discriminant Analysis (FDA) was applied (Fisher, 1936). 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. Statistically, this objective function, *J*, is as follows:

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

Here, $\bar{t}_1 = \frac{1}{n_1}\Sigma_{i=1}^{n_1} t_{1,i}$ and $\bar{t}_2 = \frac{1}{n_2}\Sigma_{i=1}^{n_2} t_{2,i}$ are the orthogonally projected means of both samples onto the direction vector and the sample variances of the projected data points are $S_1^2 = \frac{1}{n_1 - 1}\Sigma_{i=1}^{n_1}\left(t_{1,i} - \bar{t}_1\right)^2$ and $S_2^2 = \frac{1}{n_2 - 1}\Sigma_{i=1}^{n_2}\left(t_{2,i} - \bar{t}_2\right)^2$. The orthogonal projection of ith observation from the second sample, $x_{2,i}$, is $t_{2,i} = x_{2,i}^T p$, where p is the unit-length direction vector. Note that the projection coordinate, $t_{2,i}$, is often referred to as a score. Essentially, FDA is designed to best separate two groups of data while minimizing the spread out the data within each group. FDA is used to develop a multivariate model that can be used to classify between the two groups of data.

As the number of measured metabolites/ratios is 20, the next questions are (i) how many of these contribute to differences and (ii) how to independently assess the performance of the separation. To determine the most significantly contributing metabolites, all combinations involving two through ten metabolites/ratios were studied and the best 200 combinations for each total number were further evaluated. This evaluation entailed the use of a leave-one-out cross-validatory procedure (Kohavi, 1995). Leave-one-out cross-validation removes the first

observation, determining a FDA model using (1) and then applying this model to the first observation. This application is designed to determine whether this observation is correctly classified to belong to sample 1 or 2 or misclassified. Then, the second observation is left out, whilst the first observation is included for determining a second FDA model using (1). The second model is then also 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 High Risk group with ASD outcome (1st trimester, 2nd trimester and 3rd trimester) were defined as positives and the corresponding samples of the High Risk cohort with TD outcome as negatives. Similarly, when the classification between the High Risk and the Low Risk cohorts were made, the samples describing the High Risk cohort were defined as positives and the samples from the Low Risk cohort as negatives. Hypothesis testing was performed to test if an observation belongs to a cohort and the one-sided acceptance regions for a significance of $\alpha = 0.05$ was determined on the basis of a kernel density estimation of the scores for the observations of the cohort. This allowed the calculation of the number of true and false positives as well as the number of true and false negatives for the observations left out, *i.e.* independently, and with it the accuracy, specificity and sensitivity metrics and the confusion matrix. The optimum combination of metabolites/ratios was determined to be the one producing the largest accuracy.

Results

Univariate Analysis of Subgroups within the High Risk Group of Mothers

Within the High Risk group, the differences in metabolic profiles between the ASD and TD outcome subgroups were analyzed for each of the three trimesters using the appropriate statistical test with a significance level of 0.05. No significant differences were found when comparing metabolite measurements between mothers who had a child with autism and those whose child was typically-developing. Details can be found in Table S-1 in the Supplemental Materials.

Univariate Metabolite Analysis Comparing High Risk mothers with Low Risk mothers

The next set of hypotheses were tested by comparing the metabolic profiles of the Low Risk control mothers with all mothers of the High Risk subgroups, regardless of pregnancy outcome. The rationale for combining the two High Risk subgroups is that overall there are no statistically significant differences between the ASD and TD subgroups and as such they can be combined into one group. Table 3 below shows the results of the univariate analysis comparing metabolic results between High Risk mothers and Low Risk mothers for each trimester. The shaded cells represent measurements that had a statistically significant difference between the two groups (p-value   0.05 and FDR   0.1).

Table 4 below lists the means +/− standard deviations for each metabolite at each trimester separated by risk group.

For the 1st trimester data, Homocysteine, fCysteine, fCystine/fCysteine, Cys-Gly, and Nitrotyrosine have significant differences in group mean or median. Also, for the 2nd trimester data, Methionine, SAH, Homocysteine, fCystine, fCystine/fCysteine, Glu-Cys, Cys-Gly, GSSG, fGSH/GSSG, and % Oxidized GSH all have significant differences in group mean or median. Similarly, data from the 3rd trimester exhibited statistically significant differences in population mean or median for SAH, SAM/SAH, Adenosine, Homocysteine, tCysteine, fCystine/fCysteine, Glu-Cys, Nitrotyrosine, and Chlorotyrosine. Unlike the comparisons for the two subgroups within the High Risk group, there were many significant differences in mean and median between the High Risk and the Low Risk groups.

Multivariate Statistical Analysis

FDA was used to determine the classification potential of combinations of groups of metabolites to separate the data from the different groups of data (ASD vs. TD and High Risk vs. Low Risk). The optimal combinations of measurements for each classification as well as the misclassification errors after leave-one-out cross-validation have been investigated and are discussed in detail below.

Multivariate Analysis of Subgroups within the High Risk Group of Mothers.—

The combinations of metabolites that were found to provide the best separation between the ASD and TD subgroups of the High Risk group, after cross-validation was performed, are listed in Table 5 below for each time point. The misclassification errors even for these "best" combinations were in the 31%-43% range, depending upon which trimester and type of misclassification errors are considered, which indicates that no statistically significant differences can be found between the two subgroups within the High Risk mothers.

Multivariate Analysis of High Risk Mothers compared to Low Risk Mothers.—

Unlike the results for the ASD and TD subgroups within the High Risk group of mothers, multivariate analysis of the High Risk vs. the Low Risk groups returned very different results. Using multivariate analysis, Table 6 below lists the metabolic measurements that were found to result in the best separation between the High Risk and Low Risk groups as well as the misclassification errors calculated using cross-validation. In addition to the best combination of metabolites for each trimester, there was one combination of metabolites that resulted in reasonably low Type I and Type II errors for all three trimesters. This combination is marked in the table as "Overall Best Combination." The metabolites that produced the best results for each trimester are also listed in the table below as "Best Combination for Each Trimester".

The misclassification errors were low for each trimester and also reasonably low for the best combination for all three trimesters. As such, statistically significant differences of the metabolites of the transmethylation and transsulfuration pathways can be observed between the mothers in the High Risk group and those in the Low Risk group.

In order to better understand the accuracy of the metabolites resulting in the best separation of the High Risk and Low Risk group classification, confusion matrices were calculated. These confusion matrices were computed using the combination of metabolites that worked well for all three trimesters. As this combination resulted in the lowest misclassification

rates for the 3rd trimester, as can be seen in Table 6 above, only the confusion matrix for this trimester is shown in Table 7 below.

The information in the table shows how many of the members in each group are correctly classified or misclassified. For example, of the 106 mothers in Table 7 in the High Risk group for which measurements were taken during the 3rd trimester, 96 were classified as High Risk while the remaining 10 were misclassified as Low Risk. Similarly, of the 19 mothers in the Low Risk group, 17 were identified as belonging to this category while 2 were incorrectly classified as High Risk. In this table the following abbreviations are used: "TP" refers to true positive, "TN" refers to true negative, "FP" refers to false positive, "FN" refers to false negative, "PPV" refers to positive predictive value, "NPV" refers to negative predictive value, "TNR" refers to true negative rate, and "TPR" refers to true positive rate. Positive refers to the sample being classified as High Risk by the model and negative refers to the sample being classified as Low Risk by the model. True and false refer to whether or not the model's classification was correct. For example, a true positive refers to a sample from the High Risk group being classified as High Risk and a false positive refers to a sample from the Low Risk group being (incorrectly) classified as High Risk.

While only the confusion matrix for the 3rd trimester data is shown, the confusion matrices for the 1st and 2nd trimester resulted in similar, albeit slightly less accurate, predictive values. This is not unexpected as the misclassification rates for the 1st and 2nd trimester are slightly larger as shown in Table 6. Using this information from the 3rd trimester, the accuracy that a mother belonging to the High Risk group is correctly identified as such from the metabolite measurements is 90.6% (TPR from Table 7) while the accuracy of correctly identifying a mother from the Low Risk group is 89.5% (TNR from Table 7).

Discussion

Although the predictive potential of the folate-dependent transmethylation/transsulfuration metabolites for ASD or TD pregnancy outcome within the High Risk group of mothers was negative, the metabolite comparison between High Risk and Low Risk mothers indicated that there was significant potential as a predictor of whether a pregnant mother was at high or low risk of an autism pregnancy outcome. The average probability of having a child with ASD is ~1.7% (Baio, 2018) across the general population whereas mothers who have already had a child with autism (High Risk) have approximately an ~18.7% probability of having a child with ASD (Ozonoff et al., 2011). The results from our analysis, taking into account the limitations of the study as discussed below, suggest that one can predict with approximately 90% probability whether a pregnant mother is at high risk or low risk of having a child with autism by analyzing her plasma transmethylation and transsulfuration metabolites during pregnancy. While our analysis only looked at past data for comparing the High Risk to the Low Risk group, as these were determined by having had children with ASD in the past (High Risk) or not (Low Risk), the future risk levels of having a child with ASD for the two groups of mothers are based on a previous study by Ozonoff et al. (Ozonoff et al., 2011). Therefore, being able to differentiate between the two groups of mothers as shown in this study is implicative of the different risk levels of an autism pregnancy outcome.

The univariate analysis showed that there were many statistically significant differences between the High Risk and Low Risk mothers across the three trimesters. This included five for the 1st trimester, ten for the 2nd trimester, and ten for the 3rd trimester. There were two metabolite measurements that showed significant differences across all three trimesters: Homocysteine and fCystine/fCysteine. With multivariate analysis, the use of five measured metabolites across the three trimesters produced reasonably low misclassification errors and high true positive and true negative rates. There was also a combination of metabolites that produced reasonably good results for every trimester. This combination included Homocysteine, fCystine/fCysteine, Glu-Cys, fGSH, and NT. Of these five, Homocysteine and the fCystine/fCysteine redox ratio showed significant differences between the two groups across all three trimesters according to the univariate analysis. This underscores the significant contribution of these two metabolite measurements to the predictive set of five metabolites used in the multivariate classification.

The directional changes in metabolites are informative and provide insight into the abnormal metabolic status of the High Risk mothers. Homocysteine levels were consistently and significantly higher in the High Risk mothers compared to Low Risk mothers across all three trimesters in both univariate and multivariate analysis. Among Low Risk mothers, homocysteine averaged 6.98 nM/L which is consistent with the established decrease in homocysteine during normal pregnancy (Hague, 2003; Murphy, Scott, McPartlin, & Fernandez-Ballart, 2002). In contrast, the homocysteine levels averaged 8.73 in the High Risk mothers, increasing from 8.59 in the first trimester to 8.81 in the third trimester. Homocysteine lies at the intersection between folate methyl transfer and methionine transmethylation/transsulfuration metabolism (Figure 1) and an abnormal increase indicates a compromise in flux through these integrated pathways. Elevated homocysteine during pregnancy also has been associated with folate insufficiency (Bergen NE et al., 2012; Hague, 2003; Tierney, 2004), low birth weight (Murphy, Scott, Arija, Molloy, & Fernandez-Ballart, 2004), neural tube defects (Ubbink, 1995; Zhao et al., 2006), schizophrenia (Brown et al., 2007; Brown & Susser, 2008), growth retardation (Scholl & Johnson, 2000), preeclampsia (Hague, 2003) and gestational diabetes (Seghieri et al., 2003). Although not commonly practiced, it is critically important that homocysteine levels are monitored throughout pregnancy to potentially reduce the risk of these pregnancy complications which now could include autism.

By altering enzyme activity, multiple genetic polymorphisms including MTHFR (methylenetetrahydrofolate reductase), MTR (methionine synthase), MTRR (methionine synthase reductase), SAHH (S-adenosylhomocysteine hydrolase) have been shown to increase homocysteine leading to low SAM/SAH methylation capacity and epigenetically-induced abnormal gene expression during fetal development (Mathers & McKay, 2009; Ollikainen et al., 2010). SAH (S-adenosylhomocysteine) is a potent inhibitor of DNA and histone methyltransferases which are essential to regulate normal gene expression during embryonic development. The increase in SAH and decrease in SAM/SAH across all three trimesters in the High Risk mothers would be expected to reduce DNA and histone methylation leading to dysregulation of gene expression that could negatively influence neurodevelopment (Gräff & Mansuy, 2009). Elevated homocysteine can also reflect inadequate metabolite flux for glutathione synthesis and increased oxidative stress

(Mosharov, Cranford, & Banerjee, 2000; Zou & Banerjee, 2005). In High Risk mothers, elevated homocysteine was associated with elevated cystine and increased cystine/cysteine redox ratio as compared to Low Risk mothers. Cystine is the oxidized disulfide form of cysteine and an increase in plasma cystine/cysteine redox ratio reflects a pro-oxidant state in plasma that promotes pro-inflammatory cell signaling (Go et al., 2010; Iyer et al., 2009), protein thiol oxidation (Jones et al., 2004) and oxidative stress (Jones, 2006a). The cystine/ cysteine redox couple is considered to be a better indication of plasma oxidative stress than the GSH/GSSG ratio which is the dominant intracellular redox couple (Jones, 2006b). Cystathionine beta synthase (CBS), the enzyme that metabolizes homocysteine to initiate transsulfuration, is regulated by a redox sensitive cysteine that reduces CBS activity when oxidized (Niu et al., 2018). The increase in oxidized cystine/cysteine in High Risk mothers is consistent with a redox responsive decrease in CBS activity that could contribute to the increase in homocysteine (Figure 1). Other abnormalities in transsulfuration metabolism in the High Risk mothers included a decrease in Glutamylcysteine (Glu-Cys) and increase in Cysteinylglycine (Cys-Gly). Both these alterations are consistent with a compromise in GSH synthesis and recycling, respectively. Although fGSH and tGSH were maintained, GSSG was significantly increased resulting in a decrease in fGSH/GSSG redox potential and associated increase in nitrotyrosine, an indicator of protein oxidative damage. Taken together, these results indicate that folate-dependent transmethylation and transsufuration metabolism appears significantly abnormal during pregnancy in mothers at High Risk of having a child with autism compared to mothers at Low Risk of having a child with autism. A decrease in methylation potential and increase in indicators of oxidative stress are of particular concern for normal neurogenesis during fetal development (Dennery, 2007; Murphy, 2007; Parisi et al., 2017; Thompson & Al-Hasan, 2012; Yajnik & Deshmukh, 2012).

Although the data presented in Table 1 indicate that there were demographic differences between High and Low Risk mothers, it is not clear that these differences contributed to the highly significant differences in metabolic profiles between High and Low Risk group for the following reasons: 1) Although the Low Risk group were younger on average (27.9 years) than the High Risk (HR) groups (35.7 years HR-ASD and 34.6 years HR-TD), previous research indicates that glutathione and its metabolizing enzymes are relatively stable between ages of 25 and 40 and therefore age differences in this range are unlikely to have contributed to metabolic differences observed between High Risk and Low Risk groups (Al-Turk, Stohs, El-Rashidy, & Othman, n.d.); 2) Multivitamin intake during the first month of pregnancy was lower (48%) in the High Risk-ASD mothers compared to High Risk-TD mothers (67.9%); however this difference did not induce a difference in their metabolic profiles. The multivitamin intake of the Low Risk mothers (72%) was similar to the High Risk-TD mothers and therefore it is unlikely that multivitamin intake contributed to the significant metabolic differences observed between High Risk and Low Risk mothers; 3) Among mothers in the Low Risk group, 54.5% had previously had a child compared to 100% in the High Risk group. However, because there was no statistical difference in metabolic profiles among Low Risk mothers regardless whether they did or did not have a previous pregnancy, it is unlikely that a previous pregnancy influenced the metabolic results between High and Low Risk groups; 4) There were more white/Caucasian mothers in the

Low Risk group (67.9%) compared to the High Risk groups (48.3 ASD and 52.6% TD). Thus it is possible that ethnically-based genetic or epigenetic differences could potentially have affected metabolic profiles of –20% of participants. Whether or not genetic or epigenetic differences affected group metabolic profiles cannot be ascertained with the data available.

It is important to point out some other limitations of the conclusions drawn here. The sample size for the Low Risk control mothers was small due to funding limitations and may not be representative of the general population although the metabolite standard deviations were not large. In addition, we did not obtain a 3 year follow-up diagnosis for the children of the Low Risk mothers although the likelihood of ASD in this cohort is low. Another limitation may be that the High Risk and Low Risk mothers were not from the same geographical area (California and Arkansas, respectively) and different dietary habits or environmental exposures could have influenced the results. Lastly, it is not possible to know whether the maternal metabolic abnormalities we observed were directly or indirectly related to abnormal fetal neurogenesis and the developmental origins of autism.

While the results of our metabolite analysis cannot predict an autism pregnancy outcome among High Risk mothers, they do predict with reasonable accuracy whether a mother is at high risk (~18.7%) or low risk (~1.7%) of having a child with autism. If these risk estimates can be replicated in future studies, targeted therapies can be envisioned to normalize maternal folate-dependent transmethylation/transsulfuration metabolism during pregnancy and to reduce and potentially prevent the development of autism. Folic acid supplements have been shown to reduce the risk of having a child with ASD (Surén et al., 2013) and more research could be done to confirm this as a targeted therapy during pregnancy. Also, studies aimed to replicate the results could additionally include other potential biomarkers of ASD to see if these biomarkers are part of a larger set of biomarkers indicative of a higher probability of ASD diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

Al-Turk WA, Stohs SJ, El-Rashidy FH, & Othman S (n.d.). Changes in glutathione and its metabolizing enzymes in human erythrocytes and lymphocytes with age. Journal of Pharmacy and Pharmacology, 39(1), 13–16. 10.1111/j.2042-7158.1987.tb07154.x

Anderson TW, & Darling DA (1954). A test of goodness of fit. Journal of the American Statistical Association, 49(268), 765 10.2307/2281537

Andreazza AC, Kapczinski F, Kauer-Sant'Anna M, Walz JC, Bond DJ, Gonçalves CA, … Yatham LN (2009). 3-Nitrotyrosine and glutathione antioxidant system in patients in the early and late stages of bipolar disorder. Journal of Psychiatry & Neuroscience : PN, 34(4), 263–271.

Baio J (2018). Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2014. MMWR. Surveillance Summaries, 67 10.15585/mmwr.ss6706a1

Bassan FL, Kidron D, Bassan M, Rotstein M, Kariv N, Giladi E, … Harel S (2010). The effects of vascular intrauterine growth retardation on cortical astrocytes. The Journal of Maternal-Fetal & Neonatal Medicine: The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, 23(1), 595–600. 10.1080/14767050903197068

Beaudin AE, & Stover PJ (2009). Insights into metabolic mechanisms underlying folate-responsive neural tube defects: A minireview. Birth Defects Research Part A: Clinical and Molecular Teratology, 85(4), 274–284. 10.1002/bdra.20553 [PubMed: 19180567]

Bergen NE, Jaddoe VWV, Timmermans S, Hofman A, Lindemans J, Russcher H, … Steegers EAP. (2012). Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. BJOG: An International Journal of Obstetrics & Gynaecology, 119(6), 739–751. 10.1111/j.1471-0528.2012.03321.x [PubMed: 22489763]

Brown AS, Bottiglieri T, Schaefer CA, Quesenberry CP, Liu L, Bresnahan M, & Susser ES (2007). Elevated Prenatal Homocysteine Levels as a Risk Factor for Schizophrenia. Archives of General Psychiatry, 64(1), 31–39. 10.1001/archpsyc.64.1.31 [PubMed: 17199052]

Brown AS, & Susser ES (2008). Prenatal Nutritional Deficiency and Risk of Adult Schizophrenia. Schizophrenia Bulletin, 34(6), 1054–1063. 10.1093/schbul/sbn096 [PubMed: 18682377]

Chmurzynska A (2010). Fetal programming: link between early nutrition, Dna methylation, and complex diseases. Nutrition Reviews, 68(2), 87–98. 10.1111/j.1753-4887.2009.00265.x [PubMed: 20137054]

Dean OM, Buuse M van den, Bush AI, Copolov DL, Ng F, & Berk SD and M.(2009, 7 31). A Role for Glutathione in the Pathophysiology of Bipolar Disorder and Schizophrenia? Animal Models and Relevance to Clinical Practice. Retrieved May 1, 2018, from http://www.eurekaselect.com/69625/article

Dennery PA (2007). Effects of oxidative stress on embryonic development. Birth Defects Research Part C: Embryo Today: Reviews, 81(3), 155–162. 10.1002/bdrc.20098

Elmose M, Trillingsgaard A, Jørgensen M, Nielsen A, Bruhn SS, & Sørensen EU (2014). Follow-up at mid-school age (9–13 years) of children assessed for autism spectrum disorder before the age of four. Nordic Journal of Psychiatry, 68(5), 362–368. 10.3109/08039488.2013.846411 [PubMed: 24199947]

Fisher R (1936). The Else of Multiple Measurements in Taxonomic Problems. Annals of Eugenics, 7(2), 179–188.

Furness DLF, Fenech MF, Khong YT, Romero R, & Dekker GA (2008). One-carbon metabolism enzyme polymorphisms and uteroplacental insufficiency. American Journal of Obstetrics and Gynecology, 199(3), 276.e1–276.e8. 10.1016/j.ajog.2008.06.020 [PubMed: 18771981]

Go Y-M, Park FL, Koval M, Orr M, Reed M, Liang Y, … Jones DP (2010). A key role for mitochondria in endothelial signaling by plasma cysteine/cystine redox potential. Free Radical Biology and Medicine, 48(2), 275–283. 10.1016/j.freeradbiomed.2009.10.050 [PubMed: 19879942]

Gräff J, & Mansuy IM (2009). Epigenetic dysregulation in cognitive disorders. European Journal of Neuroscience, 30(1), 1–8. 10.1111/j.1460-9568.2009.06787.x [PubMed: 19508697]

Gysin R, Kraftsik R, Sandell J, Bovet P, Chappuis C, Conus P, … Do, K. Q. (2007). Impaired glutathione synthesis in schizophrenia: Convergent genetic and functional evidence. Proceedings of the National Academy of Sciences, 104(42), 16621–16626. 10.1073/pnas.0706778104

Hague WM (2003). Homocysteine and pregnancy. Best Practice & Research Clinical Obstetrics & Gynaecology, 17(3), 459–469. 10.1016/S1521-6934(03)00009-9 [PubMed: 12787538]

Hedvall A, Westerlund J, Fernell E, Holm A, Gillberg C, & Billstedt E (2014). Autism and developmental profiles in preschoolers: stability and change over time. Acta Paediatrica, 103(2), 174–181. 10.1111/apa.12455 [PubMed: 24237479]

Hertz-Picciotto I, Bennett DH, Walker C, Schmidt RJ, Oliver M, Van de Water J, … Ozonoff S (2018). Environmental Contributions to ASD Spectrum Disorder: An Introduction to the MARBLES Study, In Press.

Howsmon DP, Kruger U, Melnyk S, James SJ, & Hahn J (2017). Classification and adaptive behavior prediction of children with autism spectrum disorder based upon multivariate data analysis of markers of oxidative stress and DNA methylation. PLOS Computational Biology, 13(3), e1005385 10.1371/journal.pcbi.1005385 [PubMed: 28301476]

Howsmon DP, Vargason T, Rubin RA, Delhey L, Tippett M, Rose S, … Hahn J (2018). Multivariate techniques enable a biochemical classification of children with autism spectrum disorder versus typically-developing peers: A comparison and validation study. Bioengineering & Translational Medicine, 3(2), 156–165. 10.1002/btm2.10095 [PubMed: 30065970]

Iyer SS, Accardi CJ, Ziegler TR, Blanco RA, Ritzenthaler JD, Rojas M, … Jones DP (2009). Cysteine Redox Potential Determines Pro-Inflammatory IL-1β Levels. PLOS ONE, 4(3), e5017 10.1371/journal.pone.0005017 [PubMed: 19325908]

James SJ, Melnyk S, Jernigan S, Hubanks A, Rose S, & Gaylor DW (2008). Abnormal Transmethylation/transsulfuration Metabolism and DNA Hypomethylation Among Parents of Children with Autism. Journal of Autism and Developmental Disorders, 35(10), 1966–1975. 10.1007/s10803-008-0591-5

Jones DP (2006a). Extracellular Redox State: Refining the Definition of Oxidative Stress in Aging. Rejuvenation Research, 9(2), 169–181. 10.1089/rej.2006.9.169 [PubMed: 16706639]

Jones DP (2006b). Redefining Oxidative Stress. Antioxidants & Redox Signaling, 5(9–10), 1865–1879. 10.1089/ars.2006.8.1865

Jones DP, Go Y-M, Anderson CL, Ziegler TR, Kinkade JM, & Kirlin WG (2004). Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. The FASEB Journal, 75(11), 1246–1248. 10.1096/fj.03-0971fje

Keogh BK, Bemheimer LP, & Guthrie D (1997). Stability and change over time in cognitive level of children with delays. American Journal on Mental Retardation, 101(4), 365–373. [PubMed: 9017083]

Kohavi R (1995). A Study of Cross-validation and Bootstrap for Accuracy Estimation and Model Selection. In Proceedings of the 14th International Joint Conference on Artificial Intelligence - Volume 2 (pp. 1137–1143). San Francisco, CA, USA: Morgan Kaufmann Publishers Inc. Retrieved from http://dl.acm.org/citation.cfm?idM643031.1643047

Lowery CL, Govindan RB, Murphy P, & Eswaran H (2008). ASSESSING CARDIAC AND NEUROLOGICAL MATURATION DURING THE INTRA-UTERINE PERIOD. Seminars in Perinatology, 32(4), 263–268. 10.1053/j.semperi.2008.04.006 [PubMed: 18652925]

Mann HB, & Whitney DR (1947). On a test of whether one of two random variables is stochastically larger than the other. The Annals of Mathematical Statistics, 50–60.

Massey FJ (1951). The Kolmogorov-Smirnov Test for Goodness of Fit. Journal of the American Statistical Association, 46(253), 68 10.2307/2280095

Mathers JC, & McKay JA (2009). Epigenetics - potential contribution to fetal programming. Advances in Experimental Medicine and Biology, 646, 119–123. 10.1007/978-1-4020-9173-5_13 [PubMed: 19536670]

Mattson MP, & Shea TB (2003). Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. Trends in Neurosciences, 26(3), 137–146. 10.1016/S0166-2236(03)00032-8 [PubMed: 12591216]

Melnyk S, Fuchs GJ, Schulz E, Lopez M, Kahler SG, Fussell JJ, … James SJ (2012). Metabolic Imbalance Associated with Methylation Dysregulation and Oxidative Damage in Children with Autism. Journal of Autism and Developmental Disorders, 42(3), 367–377. 10.1007/s10803-011-1260-7 [PubMed: 21519954]

Mosharov E, Cranford MR, & Banerjee R (2000). The Quantitatively Important Relationship between Homocysteine Metabolism and Glutathione Synthesis by the Transsulfuration Pathway and Its

Regulation by Redox Changes. Biochemistry, 39(42), 13005–13011. 10.1021/bi001088w [PubMed: 11041866]

Murphy MM (2007). Homocysteine: biomarker or cause of adverse pregnancy outcome? Biomarkers in Medicine, 1(1), 145–157. 10.2217/17520363.1.1.145 [PubMed: 20477467]

Murphy MM, Scott JM, Arija V, Molloy AM, & Fernandez-Ballart JD (2004). Maternal Homocysteine before Conception and throughout Pregnancy Predicts Fetal Homocysteine and Birth Weight. Clinical Chemistry, 50(8), 1406–1412. 10.1373/clinchem.2004.032904 [PubMed: 15166109]

Murphy MM, Scott JM, McPartlin JM, & Fernandez-Ballart JD (2002). The pregnancy-related decrease in fasting plasma homocysteine is not explained by folic acid supplementation, hemodilution, or a decrease in albumin in a longitudinal study. The American Journal of Clinical Nutrition, 76(3), 614–619. 10.1093/ajcn/76.3.614 [PubMed: 12198008]

Niu W, Wang J, Qian J, Wang M, Wu P, Chen F, & Yan S (2018). Allosteric control of human cystathionine β-synthase activity by a redox active disulfide bond. Journal of Biological Chemistry, 293(1), 2523–2533. 10.1074/jbc.RA117.000103 [PubMed: 29298893]

Ollikainen M, Smith KR, Joo EJ-H, Ng HK, Andronikos R, Novakovic B, … Craig JM (2010). DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. Human Molecular Genetics, 19(21), 4176–4188. 10.1093/hmg/ddq336 [PubMed: 20699328]

Ozonoff S, Young GS, Carter A, Messinger D, Yirmiya N, Zwaigenbaum L, … Stone WL (2011). Recurrence Risk for Autism Spectrum Disorders: A Baby Siblings Research Consortium Study. Pediatrics, 128(3), e488–e495. 10.1542/peds.2010-2825 [PubMed: 21844053]

Parisi F, Rousian M, Koning AHJ, Willemsen SP, Cetin I, Steegers EAP, & Steegers-Theunissen RPM (2017). Periconceptional maternal biomarkers of one-carbon metabolism and embryonic growth trajectories: the Rotterdam Periconceptional Cohort (Predict Study). Fertility and Sterility, 107(3), 691–698.e1. 10.1016/j.fertnstert.2016.11.030 [PubMed: 28069173]

Rassin DK, Sturman JA, & Gaull GE (1981). Sulfur Amino Acid Metabolism in the Developing Rhesus Monkey Brain: Subcellular Studies of the Methylation Cycle and Cystathionine β-Synthase. Journal of Neurochemistry, 36(3), 1263–1271. 10.1111/j.1471-4159.1981.tb01726.x [PubMed: 7205268]

Rees S, Harding R, & Walker D (2008). An adverse intrauterine environment: implications for injury and altered development of the brain. International Journal of Developmental Neuroscience, 26(1), 3–11. 10.1016/j.ijdevneu.2007.08.020 [PubMed: 17981423]

Roberts G, Anderson PJ, Doyle LW, & Group, the V. I. C. S. (2010). The stability of the diagnosis of developmental disability between ages 2 and 8 in a geographic cohort of very preterm children born in 1997. Archives of Disease in Childhood, 95(10), 786–790. 10.1136/adc.2009.160283 [PubMed: 19828882]

Scholl TO, & Johnson WG (2000). Folic acid: influence on the outcome of pregnancy. The American Journal of Clinical Nutrition, 71(5 Suppl), 1295S–303S. [PubMed: 10799405]

Seghieri G, Breschi MC, Anichini R, De Beilis A, Alviggi L, Maida I, & Franconi F (2003). Serum homocysteine levels are increased in women with gestational diabetes mellitus. Metabolism, 52(6), 720–723. 10.1016/S0026-0495(03)00032-5 [PubMed: 12800097]

Smythies JR, Gottfries C-G, & Regland B (1997). Disturbances of one-carbon metabolism in neuropsychiatric disorders: A review. Biological Psychiatry, 41(2), 230–233. 10.1016/S0006-3223(96)00068-6 [PubMed: 9018395]

Storey JD (2002). A direct approach to false discovery rates. Journal of the Royal Statistical Society: Series B (Statistical Methodology), 64(3), 479–498. 10.1111/1467-9868.00346

Surén P, Roth C, Bresnahan M, Haugen M, Hornig M, Hirtz D, … Stoltenberg C (2013). Association Between Maternal Use of Folic Acid Supplements and Risk of Autism Spectrum Disorders in Children. JAMA, 309(6), 570–577. 10.1001/jama.2012.155925 [PubMed: 23403681]

Takao K, & Miyakawa T (2009). Intrauterine environment-genome interaction and children's development (4): Brain-behavior phenotyping of genetically-engineered mice using a comprehensive behavioral test battery on research of neuropsychiatric disorders. The Journal of Toxicological Sciences, 34 Suppl 2, SP293–305. [PubMed: 19571483]

Taparia S, Gelineau-van Waes J, Rosenquist TH, & Finnell RH (2007). Importance of folate-homocysteine homeostasis during early embryonic development. Clinical Chemistry and Laboratory Medicine, 45(12), 1717–1727. 10.1515/CCLM.2007.345 [PubMed: 18067451]

Thompson LP, & Al-Hasan Y (2012). Impact of Oxidative Stress in Fetal Programming [Research article], 10.1155/2012/582748

Tierney PRB and B. J. (2004, 7 31). Consequences of Elevated Homocysteine During Embryonic Development and Possible Modes of Action. Retrieved May 1, 2018, from http://www.eurekaselect.com/62591/article

Ubbink JB (1995). Is an Elevated Circulating Maternal Homocysteine Concentration a Risk Factor for Neural Tube Defects? Nutrition Reviews, 53(6), 173–175. 10.1111/j.1753-4887.1995.tb01546.x [PubMed: 7478313]

Welch BL (1947). The generalization of 'Student's' problem when several different population variances are involved. Biometrika, 34(1/2), 28–35. 10.2307/2332510 [PubMed: 20287819]

Wells PG, McCallum GP, Chen CS, Henderson JT, Lee CJJ, Perstin J, … Wong AW (2009). Oxidative Stress in Developmental Origins of Disease: Teratogenesis, Neurodevelopmental Deficits, and Cancer. Toxicological Sciences, 108(1), 4–18. 10.1093/toxsci/kfin263 [PubMed: 19126598]

Yajnik CS, & Deshmukh US (2012). Fetal programming: Maternal nutrition and role of one-carbon metabolism. Reviews in Endocrine and Metabolic Disorders, 13(2), 121–127. 10.1007/s11154-012-9214-8 [PubMed: 22415298]

Yuan T-M, Sun Y, Zhan C-Y, & Yu H-M (2010). Intrauterine infection/inflammation and perinatal brain damage: Role of glial cells and Toll-like receptor signaling. Journal of Neuroimmunology, 229(1), 16–25. 10.1016/j.jneuroim.2010.08.008 [PubMed: 20826013]

Zeisel SH (2009). Importance of methyl donors during reproduction1234. The American Journal of Clinical Nutrition, 89(2), 673S–677S. 10.3945/ajcn.2008.26811D [PubMed: 19116320]

Zhao W, Mosley BS, Cleves MA, Melnyk S, James SJ, & Hobbs CA (2006). Neural tube defects and maternal biomarkers of folate, homocysteine, and glutathione metabolism. Birth Defects Research Part A: Clinical and Molecular Teratology, 76(4), 230–236. 10.1002/bdra.20240 [PubMed: 16575882]

Zou C-G, & Baneijee R (2005). Homocysteine and redox signaling. Antioxidants & Redox Signaling, 7(5–6), 547–559. 10.1089/ars.2005.7.547 [PubMed: 15890000]

Highlights

- Metabolites from blood samples from pregnant mothers who have had a child with autism before (High Risk – 18.7%) and pregnant mothers who have not (Low Risk – 1.7%) were analyzed.

- High Risk mothers were further divided into two subgroups based on the presence (ASD) or absence (TD) of a diagnosis of autism at age 3 of the yet unborn child.

- Metabolites did not show significant differences among the two subgroups (ASD vs. TD) of the High Risk group.

- Significant differences exist in the metabolites of the High Risk vs. the Low Risk mothers.
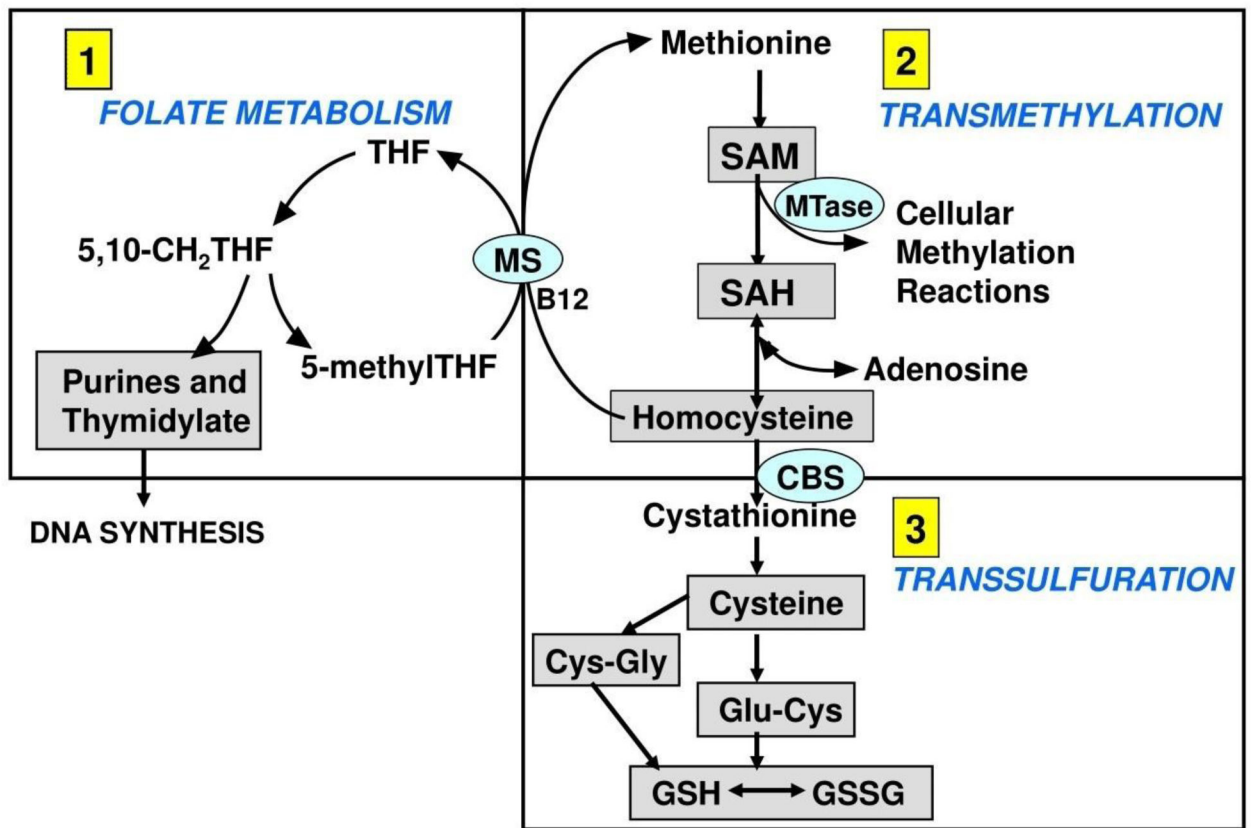
Figure 1:

Diagram of tetrahydrofolate (THF) – dependent methionine transmethylation and transsulfuration for glutathione (GSH) synthesis. THF is the metabolically active form of folate that is converted to 5-methylTHF, the primary methyl donor for methionine transmethylation. The essential amino acid methionine is regenerated and conserved by the B12-dependent transfer of a methyl group from 5-methyl THF to homocysteine in the central methionine synthase (MS) reaction. Methionine is then activated to S-adenosylmethionine (SAM), the methyl donor for multiple essential methyltransferase (MTase) reactions. The ratio of the methyl-donor SAM to the product-inhibitor S-adenosylhomocysteine (SAH) is a reflection of transmethylation pathway efficiency and cellular methylation potential. The reversible hydrolysis of SAH to homocysteine and adenosine by the SAH hydrolase (SAHH) reaction completes the methionine cycle. Homocysteine can then be remethylated to methionine or irreversibly removed from the methionine cycle by cystathionine beta synthase (CBS). This reaction initiates the transsulfuration pathway for the synthesis of cysteine and glutathione. GSH is the active reduced form of glutathione and GSSG is the inactive oxidized form. Glutamylcysteine (Glu-Cys) is the metabolic precursor for GSH and Cystinylglycine (Cys-Gly) is an intermediate in an alternate pathway for GSH synthesis.

Table 1:

Summary of the available demographic data comparing High Risk mothers with Low Risk mothers. The data for the High Risk group is subcategorized by outcomes of the children after 3 years of age as ASD (Autism Spectrum Disorder) and TD (Typically Developing). The subcategory Developmental Delay is not listed here as the data were not used for analysis. Other than age in years, values are presented as number of participants in each category with percentage in parentheses.

	High Risk ASD n =29	High Risk TD n = 78	Low Risk n = 25
Age (years)	35.7 ± 4.8	34.6 ± 4.7	27.9 ± 4.5
White/Caucasian	14 (48.3)	41 (52.6)	15 (68.2)
Non-white	15 (51.7)	37 (47.4)	7 (31.8)
Hispanic	5 (17.2)	13 (16.7)	0 (0)
Previous Pregnancy	29 (100)	78 (100)	12 (54.5)
Prenatal Vitamins first month of pregnancy	14 (48.3)	53 (67.9)	16 (72)

Table 2:

Summary of the number of measurements for each group and time point. Note that it was not possible to collect data from all of mothers during any one trimester. The data for the High Risk group is subcategorized by outcomes of the children after 3 years of age as ASD (Autism Spectrum Disorder) and TD (Typically Developing).

Groups	Time-points	Number of Mothers (separated by group)	Total Number of Mothers at each trimester
High Risk Group	1st Trimester	**ASD:** 18 **TD:** 35	53
	2nd Trimester	**ASD:** 28 **TD:** 77	105
	3rd Trimester	**ASD:** 29 **TD:** 77	106
Low Risk Group	1st Trimester	14	14
	2nd Trimester	22	22
	3rd Trimester	19	19

Table 3:

Hypothesis test results for High Risk vs. Low Risk mothers for metabolic measurements taken during each trimester. "t=" refers to the Student's t-test, "W*" refers to the Welch's test without the normality criteria being met, and "MW" refers to the Mann-Whitney U test. Significance is determined by p-value   0.05 and FDR   0.1.

Metabolite	1st Trimester			2nd Trimester			3rd Trimester		
	Test	p-value	FDR	Test	p-value	FDR	Test	p-value	FDR
Methionine	t=	0.0436	0.4702	MW	0.0013	0	MW	0.0508	0.620
SAM	MW	0.7170	1	MW	0.4541	1	MW	0.5520	1
SAH	t=	0.0950	0.9174	W	1.52E-04	0	MW	2.04E-04	0
SAM/SAH	MW	0.1139	0.9671	MW	0.2502	1	MW	7.43E-04	0
Adenosine	t=	0.2179	0.9987	W	0.0104	0.169	W	0.0071	0.0102
Homocysteine	t=	0.0050	0	MW	2.62E-06	0	MW	3.75E-06	0
tCysteine	MW	0.1747	0.9982	MW	0.5324	1	W	6.14E-04	8.38e-04
fCystine	W	0.0295	0.1998	W	4.22E-09	0	W	3.74E-09	0
fCysteine	W	0.0095	0.0707	W	0.2626	1	W	0.6428	1
fCystine/fCysteine	W*	2.50E-06	0	W	1.67E-06	0	MW	0.0042	0
Glu-Cys	t=	0.1805	0.9964	MW	0.0054	5.86e-06	MW	4.59E-04	0
Cys-Gly	t=	0.0082	0.0051	W	0.0088	6.09e-04	t=	0.0446	0.365
tGSH	t=	0.2426	0.9997	MW	0.8136	1	MW	0.6749	1
fGSH	MW	0.3920	0.9950	t=	0.5139	1	MW	0.3750	1
GSSG	W	0.0067	0.3535	MW	0.0022	0	W	0.0439	0.649
tGSH/GSSG	MW	0.6828	1	MW	0.0782	0.955	t=	0.1121	0.986
fGSH/GSSG	W	0.6736	1	MW	0.0243	0.015	W	0.0340	0.158
Nitrotyrosine	MW	0.0151	0.0160	MW	0.0412	0.235	MW	0.0036	0
Chlorotyrosine	MW	0.7402	1	W*	0.0320	0.386	W*	0.0279	0.077
% Oxidized GSH	W	0.1430	0.9948	W	0.0084	0	MW	0.0954	0.989

Table 4:

The means of the data for each group ± the standard deviations of the data split up by time point (1st trimester, 2nd trimester, and 3rd trimester) and subgroup (High Risk and Low Risk). The shaded cells refer instances with in a significance differences in mean/median as detailed in Table 2. The parentheses indicate the units of concentration. The ratios and percentage calculations are unit-less.

Metabolite	1st Trimester		2nd Trimester		3rd Trimester	
	High Risk	Low Risk	High Risk	Low Risk	High Risk	Low Risk
Methionine (μM)	23.2±5.5	20.0±4.3	21.8±4.3	18.9±3.4	22.0±4.6	19.8±3.4
SAM (μM)	55.5±12	55.4±8.8	54.9±10.3	52.7±7.4	53.6±9.8	54.7±6.0
SAH (μM)	25.8±3.6	24.0±3.1	26.2±4.0	23.8±2.1	26.7±4.2	23.2±2.4
SAM/SAH	2.20±0.57	2.34±0.44	2.14±0.50	2.24±0.42	2.05±0.50	2.38±0.35
Adenosine (μM)	0.234±0.050	0.216±0.045	0.240±0.051	0.217±0.033	0.240±0.047	0.215±0.032
Homocysteine (μM)	8.60±1.8	7.15±1.1	8.80±1.9	6.99±0.88	8.81±1.7	6.80±1.3
tCysteine (μM)	248±29	260±21	240±31	234±21	247±29	231±15
fCystine (μM)	24.6±4.0	23.3±1.0	25.1±4.2	22.0±1.3	25.4±4.5	22.3±1.0
fCysteine (μM)	22.7±3.2	24.4±1.6	22.8±3.5	22.2±2.0	23.0±3.6	22.7±1.8
fCystine/fCysteine	1.10±0.17	0.957±0.055	1.11±0.18	0.994±0.061	1.12±0.20	0.985±0.070
Glu-Cys (μM)	1.69±0.21	1.77±0.19	1.66±0.22	1.80±0.21	1.65±0.19	1.84±0.26
Cys-Gly (μM)	47.0±6.4	41.8±6.3	43.0±7.5	39.4±5.1	44.8±7.1	41.3±5.7
tGSH (μM)	6.28±1.2	5.85±1.3	6.12±1.1	6.16±1.1	6.09±1.2	6.29±1.5
fGSH (μM)	1.781±0.29	1.687±0.16	1.703±0.19	1.676±0.15	1.710±0.26	1.744±0.18
GSSG(μM)	0.229±0.048	0.205±0.022	0.229±0.041	0.206±0.026	0.235±0.036	0.221±0.026
tGSH/GSSG	28.2±8.0	28.7±6.2	27.7±7.7	30.3±6.6	26.1±6.3	28.7±6.7
fGSH/GSSG	8.14±2.3	8.31±1.0	7.73±1.9	8.22±1.1	7.42±1.5	7.95±0.86
Nitrotyrosine (nM)	42.5±10	35.5±6.3	45.8±15	38.0±6.3	49.7±16	39.2±7.0
Chlorotyrosine (nM)	32.1±11.0	29.9±4.5	33.1±13	29.5±5.1	35.2±14	31.3±4.5
% Oxidized GSH	0.208±0.048	0.196±0.018	0.213±0.037	0.198±0.019	0.218±0.036	0.202±0.018

Table 5:

Misclassification errors calculated by cross-validation using the specified combinations of metabolites. These combinations were found by choosing the combination that resulted in the lowest Type I/Type II errors.

	ASD vs. TD	
	Errors	**Metabolites**
1st Trimester	Type I = 31.43% Type II = 38.89%	Homocysteine, fCysteine, Cys-Gly, fGSH, tGSH/GSSG
2nd Trimester	Type I = 41.56% Type II = 42.86%	SAH, Homocysteine, fCystine, Glu-Cys, tGSH
3rd Trimester	Type I = 38.96% Type II = 41.38%	fCystine, fCystine/fCysteine, Glu-Cys, Cys-Gly, % Oxidized GSH

Table 6:

Misclassification errors calculated by cross-validation using the specified combinations of metabolites. Columns 2 and 3 refer to the Overall Best Combination while columns 4 and 5 list the best combination of metabolites for each trimester, which varies for each trimester.

	Overall Best Combination		Best Combination for Each Trimester	
	Errors	**Metabolites**	**Errors**	**Metabolites**
1st Trimester	Type I = 14.29% Type II = 7.55%		Type I = 7.14% Type II = 11.32%	SAH, Homocysteine, fCystine/fCysteine, fGSH, NT
2nd Trimester	Type I = 13.64% Type II = 17.14%	Homocysteine, fCystine/fCysteine, Glu-Cys, fGSH, NT	Type I = 9.09% Type 11= 13.33%	Homocysteine, fCystine, Glu-Cys, tGSH, NT
3rd Trimester	Type I = 10.53% Type II = 9.43%		Type I = 10.53% Type II = 6.60%	Methionine, SAM/SAH, fCystine/fCysteine, Glu-Cys, NT

Table 7:

The cross-validated confusion matrix for separation between the High Risk and Low Risk groups for the 3rd trimester measurements. TPR = TP/(TP+FN) is the true positive rate, TNR = TN/(TN+FP) is the true negative rate, PPV = TP/(TP+FP) is the positive predictive value, and NPV = TN/(TN+FN) is the negative predictive value.

		Actual		
		High Risk	**Low Risk**	
	High Risk	TP = 96	FP = 2	PPV = 0.97959
Predicted	**Low Risk**	FN= 10	TN= 17	NPV = 0.62963
		TPR = 0.90566	TNR = 0.89474	