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Abnormal transmethylation/transsulfuration metabolism and DNA hypomethylation among parents of children with autism

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Abstract

An integrated metabolic profile reflects the combined influence of genetic, epigenetic, and environmental factors that affect the candidate pathway of interest. Recent evidence suggests that some autistic children may have reduced detoxification capacity and may be under chronic oxidative stress. Based on reports of abnormal methionine and glutathione metabolism in autistic children, it was of interest to examine the same metabolic profile in the parents. The results indicated that parents share similar metabolic deficits in methylation capacity and glutathione-dependent antioxidant/detoxification capacity observed in many autistic children. Studies are underway to determine whether the abnormal profile in parents reflects linked genetic polymorphisms in these pathways or whether it simply reflects the chronic stress of coping with an autistic child.

Keywords

Autism; homocysteine; glutathione; DNA methylation; parents

INTRODUCTION

Autism is a behaviorally-defined neurodevelopmental disorder that is usually diagnosed in early childhood and is characterized by impairment in reciprocal communication and speech, social withdrawal, and repetitive hyper-focused behaviors. The 10-fold increase in autism diagnosis in the last decade now affecting ~1 in 150 children in the US has justifiably raised great public health concern (CDC 2005). Although both genetic and environmental factors are thought to be involved in the genesis of autism, none as yet have been reproducibly identified. Metabolic dysfunction has not been extensively studied in autistic children despite the fact that chronic biochemical imbalance is often a primary factor in the development of complex neurologic disease (Pennington et al. 2007;Kilbourne et al. 2007;Giordano et al. 2007).

In a recent study, we measured baseline levels of methionine transmethylation and transsulfuration metabolites in plasma from 80 autistic children (James et al. 2006). Briefly, we showed that the mean ratio of plasma S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAM/SAH ratio), an index of cellular methylation capacity, was 50%

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that in 75 age-matched control children. We also demonstrated a decrease in glutathione (GSH), the major intracellular antioxidant and an increase in oxidized glutathione (GSSG), resulting in a ~3-fold reduction in the ratio of reduced (active) GSH to oxidized (inactive) glutathione (GSH/GSSG). Glutathione precursors were also lower suggesting inadequate GSH synthesis. These new findings are of concern because they indicate a significant decrease in methylation capacity (↓SAM/SAH) and redox potential (↓GSH/GSSG) and an increase in oxidative stress (↑GSSG). Evidence from several other laboratories similarly indicates that biomarkers of oxidative stress may be increased in some autistic children. (Ming et al. 2005; Chauhan et al. 2004; Sogut et al. 2003; Zoroglu et al. 2004; Yao et al. 2006b)

Figure 1 is an overview of the three interdependent pathways involved in folate-dependent methionine transmethylation and transsulfuration. Pathway 1 is the folate cycle, pathway 2 is the methionine cycle, and Pathway 3 is the transsulfuration pathway leading to glutathione synthesis. The functional importance of these pathways is underscored by their essentiality for error-free DNA synthesis (Pathway 1); for cellular methylation reactions including DNA, RNA, protein, and phospholipid methylation (Pathway 2); and for the maintenance of cellular redox and detoxification capacity (Pathway 3). The GSH/GSSG ratio reflects the redox potential of the intracellular environment which is critical for maintenance of normal redox signaling, antioxidant and detoxification capacity (Wu et al. 2004;Filomeni et al. 2002;Pastore et al. 2003). Glutathione is present in millimolar concentrations inside the cell and is the major determinant of intracellular redox homeostasis and cell detoxification/antioxidant capacity. Cell functions affected by glutathione deficit include cell proliferation (e.g., immune function, DNA synthesis and repair), essential methylation (e.g., DNA, RNA, protein, phospholipid, neurotranmittors, creatine) and redox homeostasis (e.g., cell signaling, detoxification, stress response, cell cycle progression, and apoptosis).

DNA methylation is a post-translational modification of DNA that orchestrates a wide variety of functions including differentiation, tissue-specific gene expression, chromatin structure, imprinted gene expression, silencing of parasitic DNA, and X-chromosome inactivation (Razin 1998; Reik and Dean 2001; Robertson and Jones 2000; Hermann et al. 2004). Folate-dependent transmethylation metabolism is integrally involved in establishing and maintaining patterns of DNA methylation (Friso and Choi 2002; Niculescu and Zeisel 2002). DNA hypomethylation is an “epigenetic” modification that is associated with nutritional and genetic deficiencies in methionine metabolism (Jacob et al. 1998; Yi et al. 2000; Fuso et al. 2005; Friso et al. 2002c) aging (Friso and Choi 2002), cancer (Dreosti 1998), and toxic environmental exposures (Feil 2006a; Abdolmaleky et al. 2004). Epigenetic change refers to heritable alteration in gene expression without a change in DNA sequence. The ability of gene expression to adapt and respond to developmental cues and environmental exposures is primarily due to post-translational modifications of DNA methylation and histone methylation/acetylation that alter transcription factor accessibility (Abdolmaleky et al. 2004; Feil 2006b).

The possibility that autism may involve an epigenetic component is supported by reports indicating that only 60-90% of monozygotic twins are concordant for autism (Kates et al. 2004). The fact that the concordance is not 100% strongly suggests that epigenetic DNA alterations and/or gene-environment interactions contribute to the development of autism (Beaudet 2002; Badcock and Crespi 2006). Moreover, the frequency of autism is increased in children with genetic diseases that alter DNA methylation density or function including Fragile X disease, Angelman Syndrome, and Rett Syndrome (Schanen 2006; Lopez-Rangel and Lewis 2006; Samaco et al. 2005). Impaired methylation and glutathione depletion have been documented in several neuropsychiatric disorders including schizophrenia (Regland et al. 1994; Yao et al. 2006a), bipolar disorder (Kuratomi et al. 2007; Frey et al. 2007), and autism (James et al. 2004; James et al. 2006). Based on these considerations and the abnormal transmethylation and transsulfuration metabolites observed in children with autism, it was of

considerable interest to examine these pathways in the parents of autistic children as well as their global DNA methylation status.

METHODS

Study Participants

Case parents were mothers and fathers of children who had been diagnosed with Autistic Disorder based on *Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (*DSM-IV) and the Childhood Autistic Rating Scales (CARS). A total of 86 parents participated in the study consisting of 46 mothers and 40 fathers. Of the 86 parents, 72 were mother-father pairs (36 pairs), and ranged in age from 21 to 45 years (mean age ± SD: 30 ± 5). The ethnicity distribution among case parents was 87% White, 9% Asian, and 4% African American. Control parents for the metabolic study consisted of 200 mothers with a mean age of 28 (range 17-43 years) who were control participants in an ongoing case-control study of maternal risk factors for congenital heart defects (Hobbs et al. 2005a). The ethnicity among the control parents was 75% White, 7% Hispanic, 16% African American, and 2% Asian. All case and control parents were Arkansas residents. Although over-the-counter multivitamin supplement intake was significantly higher among control compared to case mothers (51% vs. 20%), metabolite levels were not significantly altered by vitamin intake within either group ($p>0.05$ for all metabolites). The protocol was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences and all participants signed informed consent.

Sample treatment and HPLC methodology

Fasting blood samples were collected before 9:00 am into EDTA-Vacutainer tubes and immediately chilled on ice before centrifuging at 4000 × g for 10 minutes at 4°C. Aliquots of plasma were transferred into cryostat tubes and stored at -80°C for approximately 2-4 weeks until extraction and HPLC quantification. For the determination of total homocysteine (tHcy), methionine, cysteine, and total glutathione, 50 μl freshly prepared 1.43 M sodium borohydride solution containing 1.5 μM.

EDTA, 66 mM NaOH and 10 μl isoamyl alcohol were added to 200 μl plasma to reduce all sulfhydryl bonds and the samples were incubated at 40°C in a shaker for 30 minutes. To precipitate proteins, 250 μl ice cold 10% meta-phosphoric acid was added, mixed well, and the sample was incubated for an additional 10 minutes on ice. After centrifugation at 18,000 × g for 15 minutes at 4°C, the supernatant was filtered through a 0.2 μm nylon membrane filter (PGC Scientific, Frederic, MD) and a 20 μl aliquot was injected into the HPLC system.

For determination of SAM, SAH and free oxidized (GSSG) and free reduced (GSH) glutathione, 100 μl of 10% meta-phosphoric acid was added to 200 μl plasma to precipitate protein; the solution was mixed well, and incubated on ice for 30 minutes. After centrifugation for 15 minutes at 18,000 g at 4°C, supernatants were passed through a 0.2 μm nylon membrane filter and 20 μl was injected into the HPLC system.

The methodological details for HPLC elution and electrochemical detection have been described previously (Melnyk et al. 2000; Melnyk et al. 1999). The analyses were performed using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C_{18} column (3 μm; 4.6 x 150 mm, MCM, Inc., Tokyo, Japan) obtained from ESA, Inc. (Chemsford, MA). The plasma extract was directly injected onto the column using Beckman Autosampler (model 507E). All plasma metabolites were quantified using a model 5200A Coulochem II and CoulArray electrochemical detection systems (ESA, Inc., Chelmsford, MA) equipped with a dual analytical cell (model 5010), a 4 channel analytical cell (model 6210) and a guard cell (model 5020). The concentrations of plasma metabolites were calculated from

peak areas and standard calibration curves using HPLC software. Although it was not technically feasible to run all case and control samples simultaneously, the -80°C storage interval was consistently between 2 and 4 weeks to minimize potential metabolite interconversion.

HPLC determination of 5-methylcystosine and total cystosine in DNA

Genomic DNA was extracted from primary lymphocytes using the Puregene Blood Kit from Gentra Systems, Inc. (Minneapolis, MN). RNase A (Sigma, St. Louis, MO) was added to a final concentration of 0.02 mg/mL and DNA incubated at 37°C for 15 minutes. The purified DNA was digested into component nucleotides using Nuclease P_1, snake venom phosphodieasterase, and alkaline phosphatase as previously described (Friso et al. 2002a). Briefly, DNA was denatured by heating for 3 minutes at 100° C and rapidly chilled in an ice water bath. One-tenth volume of 0.1 M ammonium acetate, pH 5.3, was added to 2 units of nuclease P1 (Sigma, St. Louis, MO) for every 0.5 A_{260} unit of DNA and the mixture incubated at 45°C for 2 h. Subsequently, 1/10 volume of 1 M ammonium bicarbonate and 0.002 units of venom phophodiesterase I (Sigma, St. Louis, MO) were added and the mixture incubated for 2 h at 37° C. To the mixture, 0.5 units of alkaline phosphatase (Sigma, St. Louis, MO) was then added and the incubation continued for an additional hour. The digested nucleotides were stored at - 20°C until HPLC analysis.

Statistical Analysis

Case-control differences were calculated using the Student t test with statistical significance set at $p \leq 0.05$. Linear regression analysis was used to calculate the correlation coefficients between selected metabolites and level of 5-methylcytosine using software within Microsoft Excel. Odds ratios (OR) for case compared to control mothers were calculated as an estimate of association between metabolite levels and the relative risk of being a mother of an autistic child. Throughout, a two-tailed p value of 0.05 was interpreted as a statistically significant difference.

RESULTS

Plasma transmethylation metabolite levels among cases and controls

The data presented in Table 1 indicate that plasma levels of methionine and SAM were not statistically different among case parents and controls. However, the mean levels of homocysteine and SAH were significantly elevated in case mothers compared to control mothers. Statistical evaluation of case fathers was not possible because blood samples from control fathers were not available from this cohort. Elevations in mean homocysteine and/or SAH levels were not statistically different between case mothers and fathers. [Table 1]

Because SAH is a potent product inhibitor of the DNA methyltransferase, the results were further stratified by SAH levels. Previous studies have shown that SAH levels >30 μMol/L are associated with significant DNA hypomethylation (Yi et al. 2000). As shown in Table 1, among the 200 controls, 14.9% had SAH levels > 30 μMol/L whereas 56.4% of the 86 case parents had SAH levels >30 μMol/L. Both mother and father contributed a blood sample in 72 of the 86 participants (36 pairs). In 39% of the parent pairs (14/36), both parents had SAH levels >; 30 μMol/L. Whereas mean SAM levels were not different between cases and controls, the ratio of SAM/SAH among the case parents was significantly decreased primarily reflecting the increase in SAH levels. Figure 2 demonstrates the strong and significant negative correlation between SAH levels and the SAM/SAH ratio among case parents ($r = -0.80$; $p<0.001$).

Plasma transsulfuration metabolite levels among cases and controls

Total GSH reflects combined protein-bound and free GSH after reduction of disulfide bonds whereas free GSH reflects GSH remaining after protein precipitation. Relative to control levels, parents of autistic children were found to have significant decreases in both total and free GSH levels and in the GSH/GSSG ratios whereas the levels of the oxidized disulfide GSSG were significantly increased (Table 2). Cysteine levels among the case parents were not significantly different from controls. Figure 3 shows a strong and significant correlation between plasma total GSH and free GSH among case parents ($r=0.88$; $p<0.001$). [Table 2]

DNA methylation and metabolite correlations

The percent 5-methylcytosine/total cytosine in digested DNA from case and control parents is presented in Table 3. Data are presented for combined parents because there is no known gender effect on DNA methylation. DNA from parents with SAH levels >30 μMol/L was more hypomethylated than parents with normal SAH levels (5.9% vs. 7.0% 5-methylcytosine/cytosine; $p < 0.001$). There was no statistical difference in DNA methylation between parents or controls who had SAH levels <30 μMol/L. The scatterplot in Figure 4 shows the negative correlation between SAH and % 5-methylcytosine in DNA ($r = -0.43$; $p<0.01$) consistent with the inhibitory effect of SAH on the DNA methyltransferase. There was no correlation between 5-methylcytosine and SAM or homocysteine (data not shown). [Table 3]

SAM/SAH and GSH/GSSG and maternal odds ratios

The data in Table 4 present the calculated odds ratios (OR) and 95% confidence intervals (CI) associated with maternal SAH levels and ratios of SAM/SAH and GSH/GSSG independently and combined. An elevation in SAH >30 μmol/L or a SAM/SAH ratio <2.5 increased the likelihood of being a mother with an autistic child by 7.3-fold and 10.7-fold, respectively. In the transsulfuration pathway, mothers whose GSH/GSSG ratio was < 20 had a 15.2-fold increase in the likelihood of being a mother of a child with autism. Most remarkably, among mothers who exhibited *both* SAM/SAH ratio <2.5 and a GSH/GSSG ratio <20 (41% of mothers), the odds ratio was 46. Odds ratios were not calculated for case fathers because metabolic data from control fathers was not available. [Table 4]

DISCUSSION

The results of this study demonstrate for the first time that some parents of children with autism exhibit significant metabolic deficits in methylation capacity (↓SAM/SAH) and in glutathione-mediated antioxidant and detoxification capacity (↓GSH/GSSG). A remarkably similar metabolic imbalance was recently observed in many autistic children (James et al. 2004; James et al. 2006). Preliminary evidence in the autistic children suggests that genetic polymorphisms affecting enzymes in transmethylation and transsulfuration metabolic pathways may contribute, in part, to the metabolic imbalance (James et al. 2006). The observation that both parents and children with autism share a common metabolic phenotype is consistent with an underlying genetic component. A heritable metabolic phenotype would provide a convenient target in the choice of candidate genes that predispose to complex disease and these studies in autistic families are currently underway.

Abnormal levels of metabolites in the both transmethylation and transsulfuration pathways were surprisingly prevalent among case parents (Tables 1 and 2). Both parents had significant increases in mean plasma homocysteine and SAH levels and significant decreases in the SAM/SAH ratio, total and free GSH, and the GSH/GSSG ratio. Whether the parental metabolic abnormalities are genetically-based or the result of adult dietary deficiencies and/or chronic pro-oxidant environmental exposures cannot be determined from the present data. Further, because the blood samples were taken 3-7 years after the index pregnancy, it is not possible to

know whether the maternal metabolic imbalance was present during the index pregnancy and fetal development. This is a common dilemma in studies that attempt to define maternal risk factors for adverse birth outcomes. However, it is generally accepted that adult homocysteine levels and dietary patterns during the reproductive years tend to be relatively stable (Cuco et al. 2006). For example, maternal homocysteine levels measured after pregnancy were found not to differ from pre-pregnancy values (Walker et al. 1999). Glutathione levels in healthy adults plateau between 20 and 40 years of age suggesting that adult values are also relatively stable during childbearing years (Ono et al. 2001). Nonetheless, a prospective study would be required to determine whether the observed abnormal metabolic profile is present during pregnancy and whether it could provide a maternal risk factor for having a child with autism.

Accumulating evidence suggests that epigenetic alterations may be involved in the etiology of complex neuropsychiatric diseases that do not follow classic Mendelian inheritance and are influenced by gene-environment interactions (Abdolmaleky et al. 2004; Mattson 2003; Weaver et al. 2007). For example, aberrant DNA methylation in the promoter regions of CNS *reelinRLN*) and *COMT* genes has been implicated in schizophrenia and bipolar disorder (Abdolmaleky et al. 2004; Abdolmaleky et al. 2005; Abdolmaleky et al. 2006). Based on indirect evidence, several reports have speculated that epigenetic mechanisms may contribute to the development or predisposition to autism (Schanen 2006; Nakayama et al. 2006; Lopez-Rangel and Lewis 2006). Associated with the elevated homocysteine and SAH among the case parents was a significant decrease in genome-wide DNA methylation expressed as the percent 5-methylcytosine/total cytosine in DNA (Table 3). It is well established that an increase in homocysteine levels will induce the reversal of the SAH hydrolase (SAHH) reaction causing a concomitant elevation in SAH levels (James et al. 2002; Perna et al. 2003; Castro et al. 2003). In previous studies, we and others demonstrated that plasma homocysteine and SAH levels are positively associated with genome-wide DNA hypomethylation that is most likely due to product inhibition of the DNA methyltransferase by SAH (James et al. 2002; Caudill et al. 2001; Yi et al. 2000; McKeever et al. 1995). Elevated plasma homocysteine, SAH and DNA hypomethylation are common features of autoimmune disease (Richardson 2003) and as such may be contributing factors to the increased frequency of autoimmune disease in parents of autistic children. Further, dysregulation of imprinted gene expression and maternal valproic acid exposure have been associated with elevated SAH and DNA hypomethylation and both have been implicated as risk factors for autism (Ingrosso et al. 2000; Alonso-Aperte et al. 1999; Hogart et al. 2007; Ingram et al. 2000).

The finding of genome-wide DNA hypomethylation in a significant subset of autism parents with elevated SAH provides experimental data to support earlier speculation of epigenetic mechanisms that may be associated with autism. Elevated maternal homocysteine can cross the placental membrane (Guerra-Shinohara et al. 2002) and are correlated with fetal homocysteine levels(Murphy et al. 2004). Elevation in fetal homocysteine and SAH could theoretically alter fetal methylation patterns and induce inappropriate gene expression during development that could affect predisposition to autism. In a recent study, higher maternal plasma homocysteine at preconception, prior to fetal neurogenesis, was inversely associated with cognitive achievement in the offspring (Murphy et al. 2007). Also of related interest, elevated maternal homocysteine during the third trimester was recently found to be a risk factor for schizophrenia (Bleich et al. 2007). These studies raise concern that maternal homocysteine levels should be carefully monitored and controlled especially with high risk pregnancies.

The significant difference in metabolic profiles between control and case parents was an unexpected finding. Because of the differences in mean levels of SAH, SAM/SAH and GSH/GSSG ratio between case mothers and controls, odds ratios were calculated to give an indication of the association between these metabolite levels and the likelihood of being a mother of an autistic child. Among case mothers, 54% (25/46) had SAH levels >30 μmol/L or

a SAM/SAH ratio <2.5 which was associated with 7.3-fold and 10.7-fold increased risk respectively of being a mother with an autistic child. Mothers whose GSH/GSSG ratio was < 20 had a 15.2-fold increase in the likelihood of being a mother of a child with autism. Among case mothers, 41% (19/46) exhibited a simultaneous decrease in both SAM/SAH and GSH/GSSG ratios resulting in a remarkable 46-fold increase in the likelihood of being a mother of an autistic child (Table 3).

Regardless of the origin, the finding that many parents of autistic children have an abnormal metabolic profile has important health implications in that an elevation in homocysteine and SAH levels and a decrease in SAM/SAH and GSH/GSSG ratios are well-established risk factors for heart disease, autoimmune disease, structural birth defects, and neurodegenerative disease (Morrison et al. 1999; De Bree et al. 2002; Lazzerini et al. 2007; Fidelus and Tsan 1987; Hobbs et al. 2005a; Hobbs et al. 2005b; Mattson and Shea 2003; Bains and Shaw 1997). Multiple studies have demonstrated that nutritional supplementation with B vitamins can lower homocysteine and SAH levels and that antioxidant supplementation can increase glutathione levels (Brattström et al. 1998; Zaidi et al. 2005; Ullegaddi et al. 2006). If future studies determine that a similar metabolic imbalance is present before and/or during an autism pregnancy, similar intervention strategies to normalize the maternal metabolic profile could prevent placental transfer and fetal exposure to abnormal metabolite levels.

A decrease in the GSH/GSSG redox ratio has been associated with several genetic polymorphisms, chronic dietary deficiencies, and pro-oxidant environmental exposures (Hayes and Strange 2000; Lyons et al. 2000; Greene 1995; Li et al. 2007). Glutathione can also be decreased by chronic psychological stress and severe anxiety (Eskiocak et al. 2005; Chakraborti et al. 2007). The two pathways of transmethylation and transsulfuration are metabolically interdependent such that chronic deficit in glutathione will feed back to inhibit SAM synthesis (Corrales at al. 1991) and create a chronic self-perpetuating cycle that progressively decreases GSH levels. The fact that both pathways were adversely affected in many parents suggests that the initiating factors were chronic in nature.

In summary, we have uncovered a significant metabolic imbalance in transmethylation and transsulfuration pathways in many parents that is similar to the imbalance previously observed in many autistic children. Linkage studies are underway to determine whether the shared metabolic profiles have a genetic component and psychological testing is being done to determine whether the parent profile could reflect, in part, the chronic psychological stress of coping with an autistic child. Although potentially significant, the results of this study should be considered preliminary until confirmed in subsequent studies.

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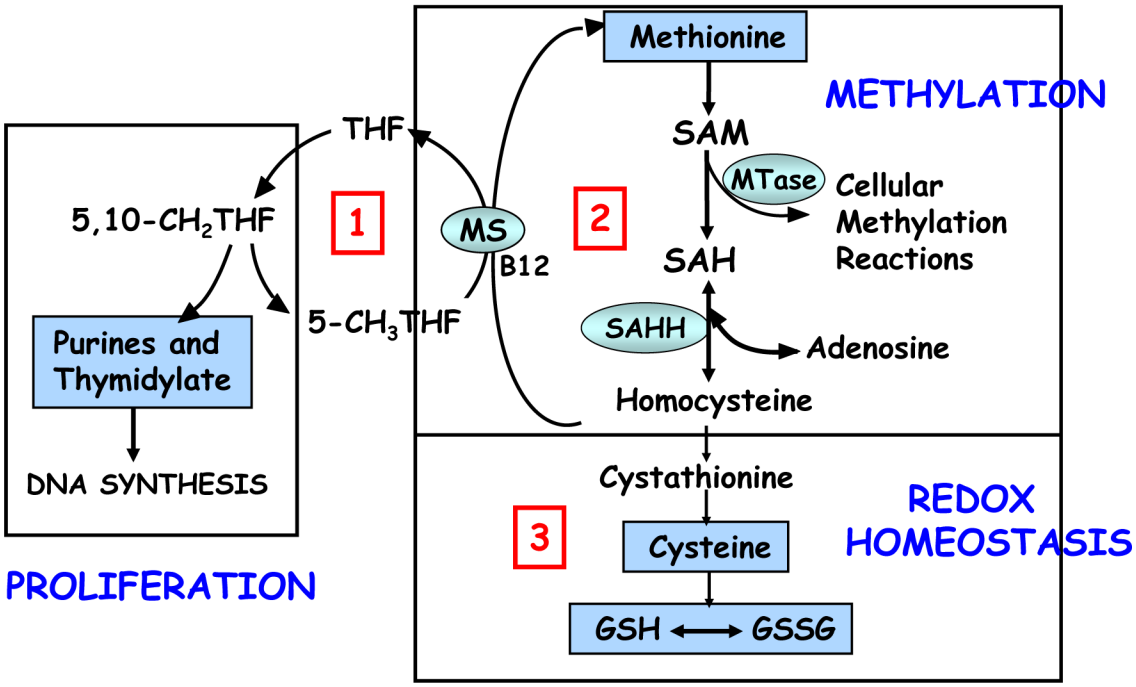

Figure 1.

A diagram of tetrahydrofolate (THF)-dependent methionine transmethylation and glutathione synthesis. The methionine cycle (transmethylation) involves the regeneration of methionine from homocysteine via the B12-dependent transfer of a methyl group from 5-methyl-tetrahydrofolate (5-CH_3THF) via the methionine synthase (MS) reaction. Methionine is then activated to S-adenosylmethionine (SAM), the methyl donor for multiple cellular methyltransferase (MTase) reactions and the methylation of essential molecules such as DNA, RNA, proteins, phospholipids, creatine, and neurotransmittors. The transfer of the methyl group from SAM results in the demethylated product S-adenosylhomocysteine (SAH). The reversible hydrolysis of SAH to homocysteine and adenosine by the SAH hydrolase (SAHH) reaction completes the methionine cycle. Homocysteine can then be either remethylated to methionine or irreversibly removed from the methionine cycle by cystathionine beta synthase (CBS). This is a one-way reaction that permanently removes homocysteine from the methionine cycle and initiates the transsulfuration pathway for the synthesis of cysteine and glutathione. Glutathione is shown in its active reduced form (GSH) and inactive oxidized disulfide form (GSSG).

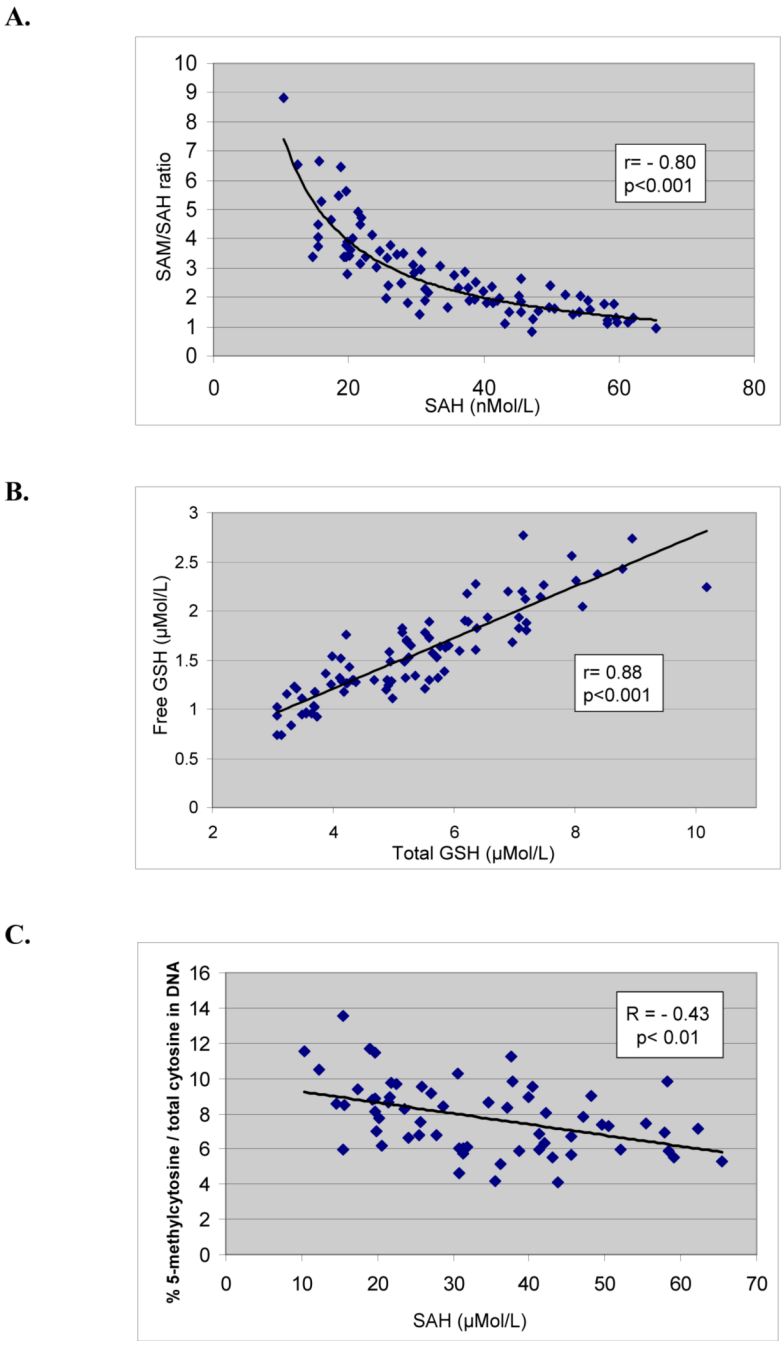

Figure 2.

A. A scatterplot showing the correlation between plasma levels of SAH and the ratio of SAM/SAH among the case parents indicating that the SAM/SAH ratio is primarily driven by the concentration of SAH. **B.** A scatterplot showing the strong correlation between free and total plasma glutathione levels among case parents. **C.** A scatterplot showing the correlation between plasma SAH levels and DNA hypomethylation (% 5-methylcytosine/total cytosine in DNA).

TABLE 1

Comparison of plasma metabolite concentrations in the transmethylation pathway between case and control parents

Plasma Transmethylation Metabolites (Mean ± SD)	Control Mothers N=200	Case Mothers N=46	Case Fathers N=40
Methionine (μMol/L)	26 ± 4.7	23 ± 4.1	26 ± 5.5
SAM (nMol/L)	83 ± 14	78 ± 20	85 ± 16
SAH (nMol/L)	23 ± 8.1	33 ± 14[a]	37 ± 15
% of subjects with SAH > 30 μMol/L	14.9%	54.3%[a]	59%
SAM/SAH Ratio	4.0 ± 1.4	2.8 ± 1.6[a]	2.7 ± 1.4
Homocysteine (μMol/L)	7.42 ± 1.7	9.8 ± 3.6[a]	10.6 ± 2.5

[a] P<0.001 Case vs. Control mothers

TABLE 2

Comparison of the plasma metabolite concentrations between case and control parents

Plasma Transsulfuration Metabolites (Mean ± SD)	Control Mothers N=200	Case Mothers N=46	Case Fathers N=40
Cysteine (μMol/L)	229 ± 22	227 ± 34	243 ± 356
Total GSH (μMol/L)	7.3 ± 1.6	5.1 ± 1.4[a]	5.7 ± 1.6
Free GSH (μMol/L)	2.6 ± 0.7	1.5 ± 0.4[a]	1.6 ± 0.5
GSSG (μMol/L)	0.24 ± 0.07	0.32 ± 0.08[a]	0.30 ± 0.09
Total GSH/GSSG Ratio	31 ± 10	17 ± 8.3[a]	20.2 ± 7.2
Free GSH/GSSG Ratio	10.7 ± 4.2	5.1 ± 2.2[a]	5.6 ± 2.2[a]

[a] Case vs. Control mothers; $p < 0.001$

TABLE 3

Comparison of the percent methylcytosine/total cytosine in DNA stratified by SAH levels

Group	N	%-Methylcytosine/Cytosine (Mean ± SD)
Combined Parents	86	6.4±1.3[b]
Controls with SAH<30 μMol/L	15	6.6±0.5[b]
Parents with SAH<30 μMol/L	38	7.0±1.5[b]
Parents with SAH>30 μMol/L	48	5.9±1.2[a]

[a] P<0.001: SAH>30 vs. SAH<30

[b] Not statistically different

TABLE 4

Odds ratios and 95% confidence intervals for the association between maternal plasma biomarkers and the likelihood of being a mother of an autistic child

Stratified Group	Control Mothers[a] N (%)	Case Mothers[b] N (%)	Odds Ratio (95% CI)
SAH >30μMol/L)	28 (14)	25 (54.3)	7.3 (3.6, 14.8)
SAM/SAH <2.5	20 (10)	25 (54.3)	10.7 (5.1, 22.5)
tGSH/GSSG <20	22 (11)	30 (65.2)	15.2 (7.2, 32.1)
SAM/SAH <2.5 and tGSH/GSSG<20	3 (1.5)	19 (41.3)	46.2 (12.8, 166.5)

[a] N=200

[b] N=46