J Autism Dev Disord (2012) 42:367–377
DOI 10.1007/s10803-011-1260-7

ORIGINAL PAPER

Metabolic Imbalance Associated with Methylation Dysregulation and Oxidative Damage in Children with Autism

Stepan Melnyk · George J. Fuchs · Eldon Schulz · Maya Lopez · Stephen G. Kahler · Jill J. Fussell · Jayne Bellando · Oleksandra Pavliv · Shannon Rose · Lisa Seidel · David W. Gaylor · S. Jill James

Published online: 26 April 2011

Abstract Oxidative stress and abnormal DNA methylation have been implicated in the pathophysiology of autism. We investigated the dynamics of an integrated metabolic pathway essential for cellular antioxidant and methylation capacity in 68 children with autism, 54 age-matched control children and 40 unaffected siblings. The metabolic profile of unaffected siblings differed significantly from case siblings but not from controls. Oxidative protein/DNA damage and DNA hypomethylation (epigenetic alteration) were found in autistic children but not paired siblings or controls. These data indicate that the deficit in antioxidant and methylation capacity is specific for autism and may promote cellular damage and altered epigenetic gene expression. Further, these results suggest a plausible mechanism by which pro-oxidant environmental stressors may modulate genetic predisposition to autism.

Keywords Autism · Oxidative stress · Metabolic · Epigenetics · Glutathione · DNA methylation

S. Melnyk · G. J. Fuchs · E. Schulz · M. Lopez · S. G. Kahler · J. J. Fussell · J. Bellando · O. Pavliv · S. Rose · L. Seidel · S. Jill James
Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, USA

D. W. Gaylor
Department of Biostatistics, University of Arkansas for Medical Sciences, Little Rock, AR, USA

S. Melnyk · G. J. Fuchs · E. Schulz · M. Lopez · S. G. Kahler · J. J. Fussell · J. Bellando · O. Pavliv · S. Rose · L. Seidel · S. Jill James (✉)
Department of Pediatrics, Arkansas Children's Hospital Research Institute, 13 Children's Way, Slot 512-41B, Little Rock, AR 72202, USA
e-mail: jamesjill@uams.edu

The metabolic pathology of autism is relatively unexplored even though metabolic imbalance is implicated in the pathogenesis of multiple other neurobehavioral disorders (Frankenburg 2007; Gysin et al. 2007; Small et al. 2000; Smythies et al. 1997). An abnormal accumulation or deficit of specific metabolites in a defined pathway can provide clues into relevant candidate genes and/or environmental exposures (Zecavati and Spence 2009). Further, the identification of precursor-product metabolite imbalance can inform targeted intervention strategies to restore metabolic balance and potentially improve symptoms of autism. We have chosen to examine key metabolites in the highly-regulated highly polymorphic pathways of folate-dependent one-carbon metabolism in autistic children and the impact of metabolic imbalance on genome-wide DNA hypomethylation and protein/DNA oxidative damage in these children. Because these pathways regulate the distribution of precursors for DNA synthesis (proliferation), DNA methylation (epigenetic regulation of gene expression) and glutathione synthesis (redox/antioxidant defense capacity), the homeostatic balance between these pathways is essential to support the rapid shifts between proliferation, differentiation and cell death that are critical determinants of normal cell programming during pre- and post-natal neurodevelopment.

Previously, we reported that many children with autism have abnormal plasma levels of metabolites in pathways of folate-dependent methionine (transmethylation) and glutathione (transsulfuration) metabolism relative to unaffected age-matched control children (James et al. 2006, 2009a). Specifically, cellular methylation capacity expressed as the mean ratio of the methyl donor S-adenosylmethionine (SAM) to the methylation inhibitor, S-adenosylhomocysteine (SAM/SAH ratio), was significantly reduced in many children with autism. A decrease in the SAM/SAH ratio has been associated with hypomethylation of DNA, RNA,

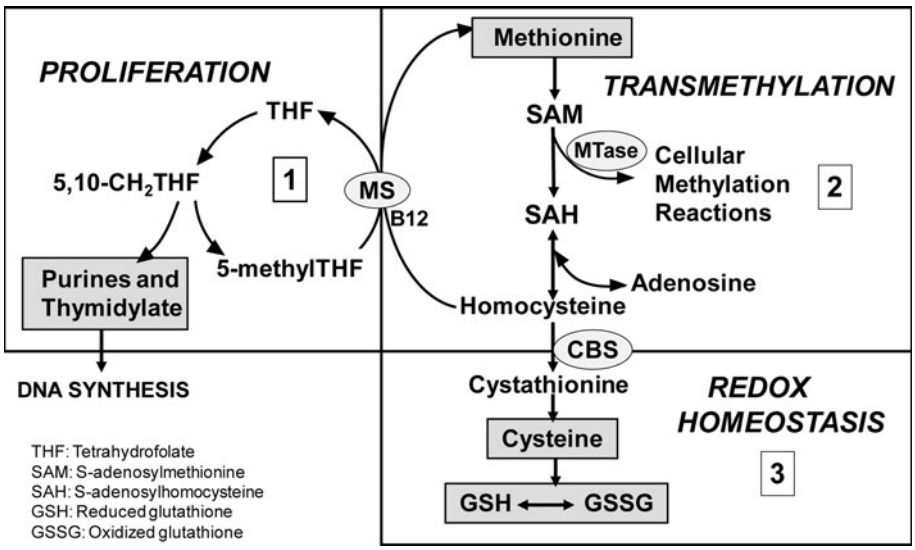

Fig. 1 A diagram of tetrahydrofolate (THF)-dependent methionine transmethylation and transsulfuration for glutathione synthesis. The methionine cycle (transmethylation) involves the regeneration of methionine from homocysteine via the B12-dependent transfer of a methyl group from 5-methyl-tetrahydrofolate (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 for the methylation of essential molecules such as DNA, RNA, proteins, phospholipids, creatine, and neurotransmitters. 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). Vital cell functions dependent on these inter-dependent pathways include proliferation (e.g., immune function, DNA synthesis and repair), methylation (e.g., DNA, RNA, proteins, phospholipids, neurotranmitters and creatine) and redox homeostasis (e.g., cell signaling, detoxification, stress response, cell cycle progression and apoptosis)

proteins, phospholipids and neurotransmitters with functional consequences in terms of gene expression, protein expression, membrane phospholipid composition and dopamine synthesis, respectively (Finkelstein 2007; Miller 2008). The mean level of reduced glutathione (GSH), the major intracellular antioxidant and mechanism for detoxification, was significantly decreased in the autistic children and the oxidized form of glutathione (GSSG) was significantly elevated resulting in a twofold reduction in the GSH/GSSG redox ratio relative to age-matched unaffected control children. Several metabolic precursors for glutathione synthesis were also lower suggesting that GSH synthesis may be insufficient. Decreased GSH/GSSG antioxidant capacity is well known to promote oxidative stress and increase vulnerability to pro-oxidant environmental exposures and oxidative damage. While these data suggest a reduced "capacity" to maintain cellular methylation and antioxidant status, it is important to note that they do not provide evidence for a functional "consequence" of the reduced capacity. In the present investigation, we report the presence of DNA hypomethylation and protein/DNA oxidative damage as potential functional consequences of reduced methylation and antioxidant capacity in these children.

To review, Fig. 1 provides an overview of the three interdependent pathways involved in folate-dependent methionine transmethylation and transsulfuration metabolism with specific biochemical details provided in the legend. Pathway 1 is the folate cycle, Pathway 2 is the methionine transmethylation cycle, and Pathway 3 is the transsulfuration pathway leading to glutathione (GSH) synthesis. The vital importance of these three interconnected pathways is underscored by their essentiality for error-free DNA synthesis (Pathway 1); for cellular methylation capacity (Pathway 2); and for the maintenance of cellular redox homeostasis (Pathway 3). The ratio of the methyl donor S-adenosylmethionine (SAM) to the product inhibitor S-adenosylhomocysteine (SAH) is a reflection of transmethylation efficiency and cellular methylation potential. Tissue-specific gene expression depends on correct promoter region DNA methylation patterns established during embryogenesis and global DNA hypomethylation can result in abnormal gene expression and genomic instability (Reik and Dean 2001; Rizwana and Hahn 1999). The GSH/GSSG ratio reflects the redox potential of the intracellular environment which is critical for maintenance of normal membrane signaling, antioxidant and detoxification capacity (Filomeni et al. 2002; Pastore et al. 2003). In

the present investigation, we evaluated global DNA methylation (as % 5-methylcytosine) to determine whether reduced methylation capacity (↓SAM/SAH) is accompanied by DNA hypomethylation and whether reduced antioxidant/detoxification capacity (↓GSH/GSSG) is associated with an increase in oxidized protein (3-nitrotyrosine) and oxidized DNA adducts (8-oxo-deoxyguanosine) in case children, paired siblings and unaffected control children.

Glutathione is a tripeptide of glutamate, glycine and cysteine and is the major intracellular redox (reduction/oxidation) buffer. The glutathione thiol/disulfide redox couple (GSH/GSSG) maintains the highly reduced intracellular microenvironment that is pivotal for effective antioxidant/detoxification capacity, redox-sensitive enzyme regulation, cell cycle progression (proliferation/differentiation), gene transcription of antioxidant response elements (ARE) and redox signaling (Biswas et al. 2006; Filomeni et al. 2002; Fratelli et al. 2005; Kwon et al. 2003; Pastore et al. 2003; Schafer and Buettner 2001). The GSH/GSSG redox status in plasma reflects intracellular hepatic metabolism where the vast majority of transsulfuration metabolism occurs. Cysteine is the rate-limiting amino acid for glutathione synthesis. The ratio of reduced cysteine (Cys) to its oxidized cystine disulfide form (CyS-S) represents the major extracellular redox couple that determines the redox status of specific cysteine residues in key regulatory cell surface proteins (Yan et al. 2009). The two major thiol/disulfide redox couples, GSH/GSSG and CyS/CyS-S, reversibly regulate the activation of key proteins by oxidative modification of sulfhydryl groups on exposed cysteine residues (Jones et al. 2004). Dynamic changes in status of glutathione and cysteine redox couples provide a "redox switch" that can regulate enzyme activation/inactivation, membrane signal transduction, cell adhesion and immune cell activation/proliferation (Biswas et al. 2006; Yan and Banerjee 2010). Oxidative stress/damage occurs when antioxidant defense mechanisms fail to counterbalance and control reactive oxygen species generated from endogenous oxidative metabolism or from pro-oxidant environmental exposures. Recent experimental studies and reviews support the hypothesis that chronic redox imbalance and resultant oxidative stress may be contributing factors to autism pathophysiology (Chauhan and Chauhan 2006; James et al. 2006; Kern and Jones 2006; Ming et al. 2005; Sogut et al. 2003; Vargas et al. 2005; Yao et al. 2006; Yorbik et al. 2002; Zoroglu et al. 2004). Based on the critical role of redox status in cell viability and function, we report for the first time the redox poise of both glutathione and cysteine redox couples as a reflection of systemic redox status in children with autism.

Because pathways of methionine transmethylation and transsulfuration are mutually interdependent, genetic or environmental perturbation of folate or methionine metabolism can indirectly impact cellular methylation capacity and glutathione synthesis. Conversely, genetic or environmentally-induced alterations in glutathione synthesis can alter flux through pathways of folate and methionine metabolism (Chan et al. 2008; Reed et al. 2008; Tchantchou et al. 2004; Vitvitsky et al. 2003). Viewed in the context of systems biology, these are core metabolic pathways that represent "hubs" for the regulation and modulation of cellular methylation, DNA synthesis and redox status in every mammalian cell. The biologic basis of autism is thought to involve gene-environment perturbations during critical developmental windows. It is therefore relevant to define the homeostasis of these polymorphic and environmentally sensitive pathways in children with autism.

In the present report, we build upon our previous findings in a new cohort of children participating in the autism *IMAGE* study (*I*ntegrated *M*etabolic *A*nd *G*enomic *E*ndeavor) that additionally provides a paired case–control comparison with unaffected siblings. Unaffected siblings represent an important new control group to determine whether the metabolic phenotype of the autistic children is specific for autism or whether it simply reflects shared genes and environmental exposures between siblings. In addition to replicating our previous report of systemic methylation and redox imbalance in plasma, we report for the first time genome-wide DNA hypomethylation (as percent 5-methylcytosine in DNA) and oxidative protein/DNA damage (as oxidized protein tyrosine derivatives and the oxidized DNA adducts) as evidence for a functional impact on epigenetic regulation and antioxidant/detoxification capacity in many children with autism.

Subjects and Methods

Participants

The autism IMAGE study (*I*ntegrated *M*etabolic *A*nd *G*enomic *E*ndeavor) is an on-going case–control study at Arkansas Children's Hospital Research Institute (ACHRI) that has recruited over 162 case and control families and is comprised of 68 case children, 40 unaffected siblings, and 54 age-matched unaffected control children. The autism case families were recruited locally after referral to the University of Arkansas for Medical Sciences (UAMS) Dennis Developmental Center and diagnosed by trained developmental pediatricians (ES, ML, JF, JB). Children 3–10 years of age with a diagnosis of Autistic Disorder as defined by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (*DSM-IV 299.0), the Autism Diagnostic Observation Schedule (ADOS), and/or the Childhood Autism Rating Scales (CARS > 30) were enrolled. The ADOS (Lord et al. 1989) and CARS

(Schopler et al. 1980) are diagnostic tools used to confirm the diagnosis and severity of autism. Children previously diagnosed with other conditions on the autism spectrum (pervasive developmental disorder-not-otherwise-specified (PDD-NOS), childhood disintegrative disorder or rare genetic diseases associated with symptoms of autism such as fragile X, Rett syndrome, or tuberous sclerosis were not included in the study. Children with chronic seizure disorders, recent infection, and high dose vitamin or mineral supplements above the RDA were excluded because these conditions are potential confounders effecting redox status. Unaffected siblings and unrelated neurotypical children ages 3–10 with no medical history of behavioral or neurologic abnormalities by parent report were the comparison groups. The unrelated neurotypical control children were was recruited by flyers placed in schools, clinics and at the University and received compensation for their participation. The mean ages of case children, unaffected siblings and unrelated control children was 5.8 ± 2.1, 5.6 ± 2.3, and 6.3 ± 2.1 years, respectively. The proportion of male children among case, sibling and control children was 85, 45, and 48%, respectively. The ethnic distribution among case families was 96% Caucasian, 2% Hispanic, and 2% Asian and among control families, the ethnic distribution was 90% Caucasian, 8% Hispanic and 2% Asian. Over-the-counter multivitamin supplement use at the time of blood draw was 35% among case children, 14% among siblings and 17% among the controls. The protocol was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences and all parents signed informed consent.

Plasma Transmethylation and Transsulfuration Metabolites, 3-Nitrotyrosine and 3-Chlorotyrosine

Fasting blood samples were collected before 9:00 am into EDTA-Vacutainer tubes and immediately chilled on ice before centrifuging at 1,300×*g* for 10 min at 4°C. Aliquots of plasma were transferred into cryostat tubes and stored at −80°C until extraction and HPLC quantification. The methodological details for HPLC elution and electrochemical detection of these plasma metabolites were developed in our laboratory and have been described previously (Melnyk et al. 1999, 2000; Shigenaga et al. 1990). The storage interval at −80°C before extraction was consistently between 1 and 2 weeks after blood draw to minimize potential metabolite inter-conversion. Between-run variation was controlled by inclusion of internal standards with each run. Plasma total folate and vitamin B12 were measured using SimulTRAC-SNB Radioassay Kit for Vitamin B12/Folate from MP Biomedical, Inc. (Orangeburg, NY).

8-Oxo-Deoxyguanosine and Percent 5-Methylcystosine/Total Cytosine in DNA

DNA was extracted from whole blood using the Puregene DNA Purification kit (Qiagen, Valencia, CA). To ~1 μg DNA, RNase A (Sigma, St. Louis, MO) was added to a final concentration of 0.02 mg/mL and incubated at 37°C for 15 min. The purified DNA was digested into component nucleotides using nuclease P_1, snake venom phosphodieasterase, and alkaline phosphatase as previously described in detail (Friso et al. 2002). DNA base separation and quantification of 5-methylcytosine and cytosine was performed with a Dionex HPLC–UV system coupled to an electrospray ionization (ESI) tandem mass spectrometer (Thermo-Finnigan LCQ) using a Phenomenex Gemini column (C18, 150 × 2.0 mm, 3 μm particle size) and expressed as percent 5-methylcytosine/total cytosine. The concentration of 8-oxo-deoxyguanosine in DNA was quantified with HPLC electrochemical detection (Helbock et al. 1998) and expressed as pmol/μg DNA.

Statistical Analyses

Metabolic data was compared using the Student's *t* test (S-Plus software, Seattle, WA) with significance set at 0.05. Given our a priori hypothesis based on previous results, correction for multiple comparisons was not implemented for case–control metabolite data. Case-sibling comparisons were done using the paired *t* test which tests the hypothesis that the mean difference between pairs is equal to zero. The paired *t* test is constructed by taking the mean difference of all observed pairs and dividing this by the standard error of all observed differences. The pairwise approach reduces variation and increases sensitivity because of the shared genes and environment between sibling pairs. Multiple regression analysis with S-Plus was used to evaluate relationships between metabolites.

Results

Transmethylation Metabolites, SAM/SAH Ratio and DNA Hypomethylation

In Table 1, the autism case children are compared to their unaffected paired sibling using the paired *t* test and cases and siblings are compared to unaffected control children using the standard Student's *t* test. Methionine is an essential amino acid that is transmethylated into homocysteine via SAM, the major cellular methyl donor. Both methionine and SAM were significantly decreased in children with autism compared to their paired unaffected siblings; however, there were no differences between siblings

Table 1 Plasma transmethylation metabolites and genome-wide DNA methylation (mean ± SD) of autistic case children, paired sibling and unaffected control children

Plasma metabolites	Cases (n = 40)	Paired sibling (n = 40)	*p* Value*	Controls (n = 54)	*p* Value**
Methionine (μmol/L)	19.8 ± 2.6[a]	22.3 ± 4.3	<0.001	23.3 ± 3.9	ns
SAM (nmol/L)	61.6 ± 8.9[a]	70.7 ± 19.4	<0.006	71.0 ± 15.6	ns
SAH (nmol/L)	20.0 ± 4.6[a]	16.9 ± 3.9	<0.001	14.8 ± 4.1	0.05
Adenosine (μmol/L)	0.16 ± 0.07	0.11 ± 0.05	<0.001	0.14 ± 0.07	ns
Homocysteine (μmol/L)	4.86 ± 1.5	4.69 ± 1.0	ns	4.68 ± 1.0	ns
Folate (ng/ml)	19.9 ± 5.1	21.6 ± 4.1	ns	19.4 ± 4.2	ns
B12 (pg/ml)	872 ± 528	719 ± 288	ns	864 ± 552	ns
SAM/SAH	3.29 ± 1.1[a]	4.4 ± 1.7	<0.001	5.08 ± 1.8	ns
DNA methylation (%5mC)	3.03 ± 0.8[a]	3.9 ± 0.7	<0.001	4.13 ± 1.0	ns

* Case-sibling comparison using paired Student's *t* test

** Sibling-control comparison using standard Student's *t* test, *ns* not significant

[a] $p < 0.001$; Case–control comparison using standard Student's *t* test

and unrelated age-matched controls. The methylation inhibitors, SAH and adenosine, were significantly increased in case children compared to their siblings. Sibling SAH levels were intermediate between case and control values and increased relative to controls. Plasma levels of homocysteine and the vitamin cofactors, folate and B12, were similar among all three groups. The SAM/SAH ratio, an indicator of methylation capacity, was decreased in children with autism compared to their siblings but not different between siblings and controls. Similarly, the percent 5-methylcytosine in DNA was decreased in case children but not in unaffected siblings and controls. Although Table 1 is limited to those case children with an unaffected sibling, the mean metabolite values of these 40 paired autism cases were not statistically different from the mean of the total IMAGE cohort of 68 case children. The children with autism were different from both siblings and unrelated controls for all metabolites except for homocysteine, folate and B12 which were not different between groups. There was no difference in the metabolite results between children who were taking over-the-counter vitamin supplements and those who did not.

Cysteine and Glutathione Metabolites and Redox Ratios

Cysteine, derived from methionine, is the rate limiting amino acid for glutathione synthesis. Total cysteine levels (protein-bound plus free) were lower in children with autism compared to their paired siblings ($p < 0.002$); however, siblings were not different from unrelated controls (Table 2).

Cystine (CyS-S), the oxidized disulfide form of cysteine, was significantly elevated in case children, but not their siblings who were also not different from controls. The free cysteine/cystine thiol redox couple (CyS/CyS-S) in plasma constitutes the major extracellular redox buffer. Shown for the first time, plasma free CyS/CyS-S redox ratio was significantly lower (more oxidized) in children with autism compared to their paired sibling whereas siblings were not

Table 2 Plasma redox metabolites (mean ± SD) of autistic case children, paired sibling and age-matched control children

Plasma metabolites	Cases (n = 40)	Paired sibling (n = 40)	*p* Value*	Controls (n = 54)	*p* Value**
Total Cysteine (μmol/L)	189 ± 21[a]	203 ± 26	<0.002	212 ± 18	ns
Free Cysteine (μmol/L)	21.6 ± 6.45	22.5 ± 5.0	ns	23.6 ± 5.3	ns
Free Cystine (μmol/L)	34.1 ± 7.5a[a]	27.1 ± 8.7	<0.001	26.4 ± 5.7	ns
Free Cysteine/Cystine	0.68 ± 0.25[a]	0.89 ± 0.25	<0.001	0.93 ± 0.27	ns
GSH (μmol/L)	1.84 ± 0.40[a]	2.06 ± 0.41	<0.001	2.58 ± 0.79	<0.001
GSSG (μmol/L)	0.23 ± 0.10[a]	0.15 ± 0.08	<0.001	0.16 ± 0.07	ns
GSH/GSSG	9.45 ± 4.08[a]	17.4 ± 10.3	<0.001	18.3 ± 8.6	ns

* Case-sibling comparison using paired Student's *t* test

** Sibling-control comparison using standard Student's *t* test, *ns* not significant

[a] $p < 0.001$; Case–control comparison using standard Student's *t* test

Table 3 Oxidized glutathione levels, protein oxidative damage and oxidative DNA damage (mean ± SD) of autistic case children, paired sibling and age-matched control children

Biomarkers of oxidative stress/damage	Cases (n = 40)	Paired sibling (n = 40)	*p* Value*	Controls (n = 54)	*p* Value**
% Oxidized GSH (2GSSG/(GSH + 2GSSG))	22 ± 8.1[a]	12.7 ± 5.9	<0.001	11.4 ± 4.1	ns
3-Nitrotyrosine (nmol/L)	143 ± 74[a]	80 ± 43	<0.001	72 ± 27	ns
3-Chlorotyrosine (nmol/L)	51 ± 18[a]	34 ± 17	<0.001	26 ± 11	0.01
8-Oxo- deoxyguanosine (pmol/mg DNA)	95 ± 35[a]	65 ± 13	<0.001	63 ± 24	ns

* Case-Sibling comparison using paired Student's *t* test

** Sibling-control comparison using standard Student's *t* test, *ns* not significant

[a] $p < 0.001$; Case–control comparison using standard Student's *t* test

different from unrelated controls. Plasma free glutathione levels were also decreased in case children compared to their paired siblings; however, mean sibling values were intermediate between cases and unaffected controls and significantly different than controls. Notably, both the major extracellular (CyS/CyS-S) and the major intracellular (GSH/GSSG) redox buffers were shifted to a more oxidized state in the children with autism compared to their siblings whereas the mean sibling values were not different from control values.

Percent Oxidized Glutathione and Protein/DNA Oxidative Damage

The percent oxidized glutathione, expressed as glutathione equivalents (2GSSG/(GSH + 2GSSG), is a comprehensive indicator of plasma and intracellular glutathione redox potential (Lenton et al. 1999). The case-sibling pair comparison was highly significantly different in contrast to the sibling-unrelated control comparison which was not different (Table 3).

Both 3-nitrotyrosine and 3-chlorotyrosine are stable oxidative post-translational modifications of protein tyrosine residues and both were significantly increased in plasma from children with autism compared to their paired siblings. In nuclear and mitochondrial DNA, 8-oxodG is a free radical-induced oxidative lesion that is widely used as a biomarker of oxidative damage. Table 3 shows the highly significant increase in leukocyte DNA 8-oxo-deoxyguanosine (8-oxo-dG) levels in children with autism compared to their siblings but no difference between unaffected siblings and unaffected control children.

Figures 2a and b are representative scatter plots that display individual data points of all 68 case and 54 unrelated control participants in the autism IMAGE study. There was a positive correlation between the two oxidized endpoints, nitrotyrosine and percent oxidized glutathione ($r = 0.50$), and a disparate distribution between cases and controls with control subjects clustered at the lower less oxidized end of the distribution (Fig. 2a). The negative correlation between glutathione concentration and oxidized nitrotyrosine levels ($r = -0.41$, Fig. 2b) is consistent with the protective antioxidant function of glutathione. Sibling values overlapped with the control values in both correlations (data not shown).

Discussion

An "endophenotype" can be a biochemical, neurologic, hormonal or immunologic biomarker that is influenced by both genes and environment and that is reproducibly associated with clinical symptoms of the disease (Gottesman and Gould 2003). As such, it can provide targeted clues to susceptibility alleles and targets of environmental vulnerability. Because an endophenotype is potentially modifiable, it could additionally provide insights into treatment strategies that can be followed longitudinally during intervention for clinical efficacy. The abnormal metabolic endophenotype we have observed in many autistic children may be due to subtle changes in gene products that regulate flux through folate and methionine dependent pathways or could reflect environmental exposures that perturb these pathways. Even small variations in gene expression and enzyme activity, if expressed chronically, can have a significant impact on downstream metabolic dynamics with functional consequences especially during pivotal developmental windows.

To determine whether the observed metabolic phenotype is specific to autism, metabolic profiles from proband-sibling pairs and unaffected control children were compared. Within our autism IMAGE cohort, the majority of metabolites in the methylation/redox pathways were significantly different between affected and unaffected siblings whereas unaffected siblings were not different from unrelated controls. These results suggest that these methylation/redox biomarkers are specific for autistic disorder and not present in unaffected siblings who share similar genes and environmental exposures. However, because the plasma samples were obtained after diagnosis, it is not

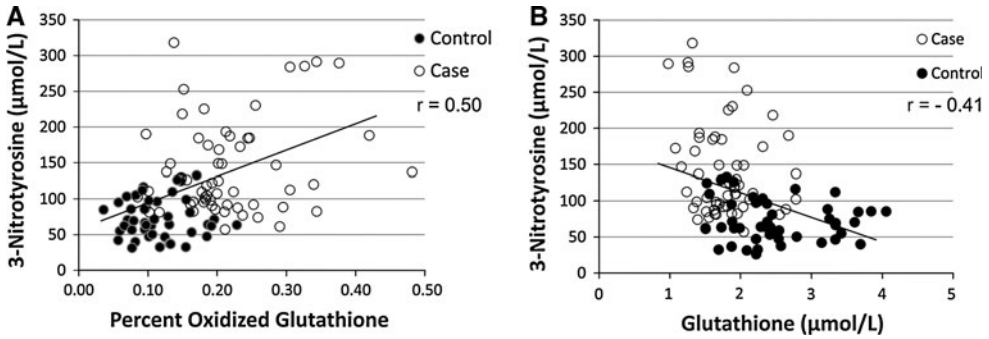

Fig. 2 **a** The positive correlation between the two oxidized endpoints, nitrotyrosine and percent oxidized glutathione ($r = 0.50$), and the disparate distribution between cases (*open circles*) and controls (*closed circles*) with control subjects clustered at the lower (less oxidized) end of the distribution. **b** The negative correlation between glutathione concentration and oxidized nitrotyrosine levels ($r = -0.41$) and is consistent with the protective antioxidant function of glutathione

possible to discern whether the observed deficits in methylation and redox capacity contribute to autism pathogenesis or are simply a reflection of on-going autism pathophysiology. Nonetheless, treatment to restore methylation and redox homeostasis has been shown to be efficacious in some children with autism and also in individuals with schizophrenia and bipolar disorder (Berk et al. 2008a, b; Dean et al. 2009; Dodd et al. 2008; Frankenburg 2007; James et al. 2009a; Strous et al. 2009). Although these studies suggest that targeted treatment can reverse the biochemical imbalance, further confirmation and randomized clinical trials are needed to determine whether restoration of metabolic balance can also improve medical and behavioral symptoms in children with autism.

The autism IMAGE study is our third independent case–control study that confirms findings of decreased plasma concentrations of methylation precursors and decreased SAM/SAH methylation potential in autistic children (James et al. 2006, 2009a). While reduced levels of metabolic precursors suggest reduced methylation capacity, here we report for the first time that genome-wide DNA methylation is also reduced in many autistic children. Although OTC vitamin use was higher in the autism group than in sibling and unaffected controls, the decrease in global DNA methylation observed was opposite of an expected effect. Further, statistical comparison of DNA methylation and plasma metabolites between children who were and were not taking OTC supplements revealed no significant differences in any group. Thus, we conclude that OTC vitamin use had no apparent effect on any of the endpoints measured. The presence of global DNA hypomethylation implies epigenetic dysregulation possibly due to reduced methyl donor (SAM) availability and/or increased product (SAH) inhibition of DNA and histone methyltransferases. A decrease in SAM/SAH methylation potential and DNA hypomethylation have been previously implicated in the etiology of cardiovascular disease, cancer, schizophrenia, autoimmunity and birth defects (Castro et al. 2003; Dunlevy et al. 2006; Li et al. 2010; Schmutte et al. 1996). Epigenetic modifications endow the genome with adaptive plasticity in gene expression in response to a changing environment and do so without altering DNA sequence. Primary epigenetic mechanisms include DNA cytosine methylation and histone methylation/acetylation that function to alter chromatin structure and transcription factor accessibility to initiate gene expression. Because DNA and histone methyltransferase reactions involve the transfer of a methyl group from SAM, epigenetics is integrally linked to transmethylation metabolism and methyl donor availability. While our observations of decreased methyl donor (SAM) and increased methyltransferase inhibitor (SAH) are consistent with epigenetic dysregulation, the functional significance of genome-wide DNA hypomethylation has yet to be defined.

Increasing evidence from both human and animal models suggest that aberrant DNA methylation during prenatal and early postnatal development might have a role in the etiology of autism (Allan et al. 2008; Jiang et al. 2004; Nagarajan et al. 2008; Samaco et al. 2005; Schanen 2006). DNA methylation is a mitotically heritable epigenetic mechanism that determines tissue-specific gene expression and silencing during pre-natal and post-natal neurodevelopment. Fetal DNA methylation patterns are established very early during embryogenesis and provide the basis for tissue-specific gene expression, allele-specific gene imprinting, X chromosome inactivation, and chromosome stability (Dean and Ferguson-Smith 2001; Reik 2007). Thus, aberrant epigenetic programming during critical periods of fetal development can result in aberrant timing of gene expression and cell differentiation that can heritably alter fetal phenotype (Zeisel 2009). In addition to mediating gene-environment interactions, epigenetic variation in gene expression might help to explain the broad

heterogeneity in symptom severity, onset and heritability within the autism spectrum. This provocative possibility requires further investigation.

Impaired methionine metabolism has been reproducibly associated with epigenetic dysregulation in several other neurobehavioral disorders (Costa et al. 2009; Graff and Mansuy 2009; Grayson et al. 2009; Krebs et al. 2009). Initial genome-wide epigenetic profiling in individuals with schizophrenia and bipolar disorder revealed DNA methylation changes that mapped to loci involving mitochondrial function, brain development as well as GABAergic and glutamatergic neurotransmission (Mill et al. 2008). Under pathologic progression, global DNA hypomethylation is often paradoxically associated with promoter-specific DNA hypermethylation and down-regulation of gene expression (Ehrlich 2002). Consistent with our observations of DNA hypomethylation in many children with autism, gene-specific alterations in DNA methylation of MeCP2 and oxytocin receptor genes have been recently reported in post-mortem autism brain (Gregory et al. 2009; Nagarajan et al. 2008). Together, these preliminary results support the possibility that epigenetic dysregulation contributes to aberrant gene expression in autism.

As indicated in Fig. 1, methionine transmethylation metabolism is integrally related to transsulfuration by providing metabolic precursors for cysteine and glutathione synthesis and in turn redox homeostasis. The autism IMAGE cohort replicates previous reports of a decrease in glutathione precursors and also a decrease GSH/GSSG redox status (James et al. 2006, 2009a). Plasma GSH/GSSG redox ratio is a reflection of intracellular redox homeostasis primarily due to the cellular export of GSSG to the plasma under conditions of intracellular oxidative stress (Eklow et al. 1981). Oxidized GSSG was significantly elevated among the autistic children compared to their siblings who were not different from unrelated controls. In addition, the IMAGE study provides new evidence for a decrease in extracellular free cysteine/cystine (CyS/CyS-S) redox poise and an increase in oxidative protein and DNA damage in many children with autism but not in their paired siblings. These findings are consistent with our hypothesis that transsulfuration metabolism in children with autism may not able to maintain redox homeostasis in the presence of chronic oxidative stress. Similar evidence of oxidative stress in blood, CSF and brain has been similarly reported in patients with schizophrenia and bipolar disorder (Andreazza et al. 2009; Dean et al. 2009). Total GSH concentration was decreased 27% in CSF and 52% in medial prefrontal cortex of schizophrenic patients (Do et al. 2000). Recently, a genetic and functional deficit in glutamylcysteine ligase (GCL), the rate limiting enzyme for glutathione synthesis, was associated with decreased GSH levels in fibroblasts derived from schizophrenic patients (Gysin et al. 2007). Although the age of onset is clearly different between schizophrenia and autism, the onset of both disorders coincides with critical time periods (infancy and adolescence) during which brain maturational processes and fine tuning of neuronal circuitry occurs (Giorgio et al. 2010; Guerri and Pascual 2010). Glutathione redox status is a pivotal determinant of oligodendrocyte and neuronal progenitor cell signaling in the branch point decision whether to differentiate, proliferate, or die (Noble et al. 2003). Thus, it is theoretically plausible that subtle redox imbalance during pivotal periods of brain cell maturation and development could negatively affect downstream brain cell trajectories, connectivity and synchronization in both schizophrenia and autism.

Plasma concentrations of oxidized protein tyrosine derivatives (3-nitrotyrosine and 3-chlorotyrosine) were increased in many of the autism IMAGE participants relative to paired siblings and control children. These post-translational modifications provide a stable biochemical footprint of oxidative stress and protein oxidative damage that can be followed longitudinally. Nitrotyrosine originates from protein tyrosines primarily via free radical attack by peroxynitrite, the highly reactive product of superoxide and nitric oxide. Chlorotyrosine is created by hypochlorous acid, a potent chlorinating oxidant derived from myeloperoxidase released by activated macrophages and neutrophils during an inflammatory response. As a marker of oxidative stress, elevated levels of nitrotyrosine have been found in alcoholics, smokers, diabetes, atherosclerosis, cystic fibrosis, and in pre-term infants (Mohiuddin et al. 2006). Within our autism cohort, nitrotyrosine was positively correlated with percent oxidized glutathione ($r = 0.50$; $p < 0.001$). Supporting our observations in plasma, a recent preliminary study found increased levels of nitrotyrosine in autism post-mortem brain (Sajdel-Sulkowska et al. 2009).

The 8-oxo-dG adduct in nuclear and mitochondrial DNA is a pre-mutagenic lesion that has been associated with oxidative DNA damage and the pathophysiology of aging, cancer and pro-oxidant environmental exposures (Pilger and Rudiger 2006). Once incorporated, 8-oxo-dG codes for error-prone DNA synthesis that promotes de novo mutations, repair-associated DNA strand breaks and genomic instability (Dahlmann et al. 2009). Elevated levels of 8-oxo-dG in DNA, plasma or urine is a commonly used biomarker for assessing oxidative DNA damage during inflammatory disease and pro-oxidant exposures. Our data indicating a significant increase in 8-oxo-dG concentration in peripheral lymphocyte DNA in children with autism is a new finding consistent with reduced glutathione-mediated antioxidant/detoxification capacity. The biological relevance of 8-oxo-dG is its established ability to induce G > T transversions which are common somatic mutations

under pro-oxidant conditions. While the functional relevance of this new finding is not clear, it provides substantial evidence for an oxidized nuclear microenvironment and DNA structural instability. In addition, it offers a possible mechanism for the multiple and variable de novo mutations that have been found to be present in children with autism but not their parents (Gauthier et al. 2009; Sebat et al. 2007; Smith et al. 2009).

Genomic instability and de novo mutations secondary to an oxidizing microenvironment can occur in parental germ cells, gestation and/or during early post-natal development in nuclear and/or mitochondrial DNA. Mitochondria are the primary source and target of reactive oxygen species and, lacking histones and DNA repair mechanisms, mitochondrial DNA is exceptionally vulnerable to oxidative damage. In an earlier study, we measured mitochondrial redox status in lymphoblastoid cells derived from autistic to control individuals and found that mitochondrial GSH/GSSG redox status was significantly more oxidized and accompanied by increased generation of reactive oxygen species (ROS) in autism compared to control cells (James et al. 2009b). These results together with the present data indicating oxidative DNA damage in primary leukocytes warrants further investigation into genomic instability, cellular and sub-cellular sequelae of oxidative stress in the autism brain.

It is generally agreed that the pathobiology of autism involves multiple and variable genetic and environmental factors that interact to increase risk of developing the disorder. Intense research effort has uncovered several potential targets of gene-environment interactions including impairments in mitochondrial function, synaptic connectivity, calcium channeling, excitation/inhibition and excessive glutamate among others. Attempts to coalesce these disparate clues into a unified conceptual framework or final common pathway have been the focus of several reviews and have yielded several plausible hypotheses that warrant further research investigation (Belmonte et al. 2004; Bourgeron 2009; Rubenstein 2010; Rubenstein and Merzenich 2003). Genetic and/or environmentally-induced alterations in folate-dependent one carbon metabolism (transmethylation and epigenetics) linked to methionine-dependent sulfur metabolism (transsulfuration and oxidative stress) offers another plausible framework that could connect some of these disparate functional correlates of autism. Epigenetic alterations and oxidative DNA damage observed in the present study are consistent with the gene-environment model proposed in the LEARn (Latent Early-life Associated Regulation) explanation for idiopathic neurobiologic diseases (Lahiri et al. 2009). The LEARn model proposes that genetic and environmental risk factors operate through alterations in DNA methylation and oxidative damage in susceptible genes resulting in altered gene expression. Unlike DNA sequence change, both redox and epigenetic alterations are dynamic adaptive responses to environmental stressors that are inherently reversible; thus, a deeper understanding of these alterations could not only provide new insights into the basic neurobiology of autism but could lead to novel targeted therapeutic strategies to treat and possibly prenatally prevent the development of autism.

Acknowledgments The authors would like to express their gratitude to the families in Arkansas affected by autism whose participation made this study possible. We also acknowledge the invaluable help of the nurses and clinicians at the Dennis Developmental Center for referral and evaluation. This research was supported, in part, with funding from the National Institute of Child Health and Development (RO1 HD051873; SJJ), the Department of Defense (AS073218P1; SJJ) and by grants from the Arkansas Children's Hospital and Arkansas Biosciences Institute (SJJ).

References

Allan, A. M., Liang, X., Luo, Y., Pak, C., Li, X., Szulwach, K. E., et al. (2008). The loss of methyl-CpG binding protein 1 leads to autism-like behavioral deficits. *Human Molecular Genetics, 17*, 2047–2057.

Andreazza, A. C., Kapczinski, F., Kauer-Sant'Anna, M., Walz, J. C., Bond, D. J., Goncalves, C. A., et al. (2009). 3-Nitrotyrosine and glutathione antioxidant system in patients in the early and late stages of bipolar disorder. *Journal of Psychiatry & Neuroscience, 34*, 263–271.

Belmonte, M. K., Cook, E. H., Jr., Anderson, G. M., Rubenstein, J. L., Greenough, W. T., Beckel-Mitchener, A., et al. (2004). Autism as a disorder of neural information processing: Directions for research and targets for therapy. *Molecular Psychiatry, 9*, 646–663.

Berk, M., Copolov, D., Dean, O., Lu, K., Jeavons, S., Schapkaitz, I., et al. (2008a). N-acetyl cysteine as a glutathione precursor for schizophrenia—A double-blind, randomized, placebo-controlled trial. *Biological Psychiatry, 64*, 361–368.

Berk, M., Ng, F., Dean, O., Dodd, S., & Bush, A. I. (2008b). Glutathione: A novel treatment target in psychiatry. *Trends in Pharmacological Sciences, 29*, 346–351.

Biswas, S., Chida, A. S., & Rahman, I. (2006). Redox modifications of protein-thiols: Emerging roles in cell signaling. *Biochemical Pharmacology, 71*, 551–564.

Bourgeron, T. (2009). A synaptic trek to autism. *Current Opinion in Neurobiology, 19*, 231–234.

Castro, R., Rivera, I., Struys, E. A., Jansen, E. E. W., Ravasco, P., Camilo, M. E., et al. (2003). Increased, homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. *Clinical Chemistry, 49*, 1292–1296.

Chan, A., Tchantchou, F., Graves, V., Rozen, R., & Shea, T. B. (2008). Dietary and genetic compromise in folate availability reduces acetylcholine, cognitive performance and increases aggression: Critical role of S-adenosyl methionine. *The Journal of Nutrition, Health & Aging, 12*, 252–261.

Chauhan, A., & Chauhan, V. (2006). Oxidative stress in autism. *Pathophysiology, 13*, 171–181.

Costa, E., Chen, Y., Dong, E., Grayson, D. R., Kundakovic, M., Maloku, E., et al. (2009). GABAergic promoter hypermethylation as a model to study the neurochemistry of schizophrenia vulnerability. *Expert Review of Neurotherapeutics, 9*, 87–98.

Dahlmann, H. A., Vaidyanathan, V. G., & Sturla, S. J. (2009). Investigating the biochemical impact of DNA damage with structure-based probes: Abasic sites, photodimers, alkylation adducts, and oxidative lesions. *Biochemistry, 48*, 9347–9359.

Dean, W., & Ferguson-Smith, A. (2001). Genomic imprinting: Mother maintains methylation marks. *Current Biology, 11*, R527–R530.

Dean, O. M., van den Buuse, M., Bush, A. I., Copolov, D. L., Ng, F., Dodd, S., et al. (2009). A role for glutathione in the pathophysiology of bipolar disorder and schizophrenia? Animal models and relevance to clinical practice. *Current Medicinal Chemistry, 16*, 2965–2976.

Do, K. Q., Trabesinger, A. H., Kirsten-Kruger, M., Lauer, C. J., Dydak, U., Hell, D., et al. (2000). Schizophrenia: Glutathione deficit in cerebrospinal fluid and prefrontal cortex in vivo. *European Journal of Neuroscience, 12*, 3721–3728.

Dodd, S., Dean, O., Copolov, D. L., Malhi, G. S., & Berk, M. (2008). N-acetylcysteine for antioxidant therapy: Pharmacology and clinical utility. *Expert Opinion on Biological Therapy, 8*, 1955–1962.

Dunlevy, L. P., Burren, K. A., Mills, K., Chitty, L. S., Copp, A. J., & Greene, N. D. (2006). Integrity of the methylation cycle is essential for mammalian neural tube closure. *Birth Defects Research. Part A, Clinical and Molecular Teratology, 76*, 544–552.

Ehrlich, M. (2002). DNA methylation in cancer: Too much, but also too little. *Oncogene, 21*, 5400–5413.

Eklow, L., Thor, H., & Orrenius, S. (1981). Formation and efflux of glutathione disulfide studied in isolated rat hepatocytes. *FEBS Letters, 127*, 125–128.

Filomeni, G., Rotilio, G., & Ciriolo, M. R. (2002). Cell signalling and the glutathione redox system. *Biochemical Pharmacology, 64*, 1057–1064.

Finkelstein, J. D. (2007). Metabolic regulatory properties of S-adenosylmethionine and S-adenosylhomocysteine. *Clinical Chemistry and Laboratory Medicine, 45*, 1694–1699.

Frankenburg, F. R. (2007). The role of one-carbon metabolism in schizophrenia and depression. *Harvard Review of Psychiatry, 15*, 146–160.

Fratelli, M., Goodwin, L. O., Orom, U. A., Lombardi, S., Tonelli, R., Mengozzi, M., et al. (2005). Gene expression profiling reveals a signaling role of glutathione in redox regulation. *PNAS, 102*, 13998–14003.

Friso, S., Choi, S. W., Dolnikowski, G. G., & Selhub, J. (2002). A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass spectrometry. *Analytical Chemistry, 74*, 4526–4531.

Gauthier, J., Spiegelman, D., Piton, A., Lafreniere, R. G., Laurent, S., St-Onge, J., et al. (2009). Novel de novo SHANK3 mutation in autistic patients. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics: The Official Publication of the International Society of Psychiatric Genetics, 150B*, 421–424.

Giorgio, A., Watkins, K. E., Chadwick, M., James, S., Winmill, L., Douaud, G., et al. (2010). Longitudinal changes in grey and white matter during adolescence. *Neuroimage, 49*, 94–103.

Gottesman, I. I., & Gould, T. D. (2003). The endophenotype concept in psychiatry: Etymology and strategic intentions. *American Journal of Psychiatry, 160*, 636–645.

Graff, J., & Mansuy, I. M. (2009). Epigenetic dysregulation in cognitive disorders. *European Journal of Neuroscience, 30*, 1–8.

Grayson, D. R., Chen, Y., Dong, E., Kundakovic, M., & Guidotti, A. (2009). From trans-methylation to cytosine methylation: Evolution of the methylation hypothesis of schizophrenia. *Epigenetics, 4*, 144–149.

Gregory, S. G., Connelly, J. J., Towers, A. J., Johnson, J., Biscocho, D., Markunas, C. A., et al. (2009). Genomic and epigenetic evidence for oxytocin receptor deficiency in autism. *BMC Medicine, 7*, 62.

Guerri, C., & Pascual, M. (2010). Mechanisms involved in the neurotoxic, cognitive, and neurobehavioral effects of alcohol consumption during adolescence. *Alcohol, 44*, 15–26.

Gysin, R., Kraftsik, R., Sandell, J., Bovet, P., Chappuis, C., Conus, P., et al. (2007). Impaired glutathione synthesis in schizophrenia: Convergent genetic and functional evidence. *PNAS, 104*, 16621–16626.

Helbock, H. J., Beckman, K. B., Shigenaga, M. K., Walter, P. B., Woodall, A. A., Yeo, H. C., et al. (1998). DNA oxidation matters: The HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *PNAS, 95*, 288–293.

James, S. J., Melnyk, S., Fuchs, G., Reid, T., Jernigan, S., Pavliv, O., et al. (2009a). Efficacy of methylcobalamin and folinic acid treatment on glutathione redox status in children with autism. *American Journal of Clinical Nutrition, 89*, 425–430.

James, S. J., Melnyk, S., Jernigan, S., Cleves, M. A., Halsted, C. H., Wong, D. H., et al. (2006). Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics, 141*, 947–956.

James, S. J., Rose, S., Melnyk, S., Jernigan, S., Blossom, S., Pavliv, O., et al. (2009b). Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism. *FASEB Journal, 23*, 2374–2383.

Jiang, Y. H., Sahoo, T., Michaelis, R. C., Bercovich, D., Bressler, J., Kashork, C. D., et al. (2004). A mixed epigenetic/genetic model for oligogenic inheritance of autism with a limited role for UBE3A. *American Journal of Medical Genetics, 131A*, 1–10.

Jones, D. P., Go, Y. M., Anderson, C. L., Ziegler, T. R., Kinkade, J. M., Jr., & Kirlin, W. G. (2004). Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. *FASEB Journal, 18*, 1246–1248.

Kern, J. K., & Jones, A. M. (2006). Evidence of toxicity, oxidative stress, and neuronal insult in autism. *Journal of Toxicology and Environmental Health. Part B, Critical Reviews, 9*, 485–499.

Krebs, M. O., Bellon, A., Mainguy, G., Jay, T. M., & Frieling, H. (2009). One-carbon metabolism and schizophrenia: Current challenges and future directions. *Trends in Molecular Medicine, 15*, 562–570.

Kwon, Y. W., Masutani, H., Nakamura, H., Ishii, Y., & Yodoi, J. (2003). Redox regulation of cell growth and cell death. *Biological Chemistry, 384*, 991–996.

Lahiri, D. K., Maloney, B., & Zawia, N. H. (2009). The LEARn model: An epigenetic explanation for ideopathic neurobiologic diseases. *Molecular Psychiatry, 14*, 992–1003.

Lenton, K. J., Therriault, H., & Wagner, J. R. (1999). Analysis of glutathione and glutathione disulfide in whole cells and mitochondria by postcolumn derivatization high-performance liquid chromatography with ortho-phthalaldehyde. *Analytical Biochemistry, 274*, 125–130.

Li, Y., Liu, Y., Strickland, F. M., & Richardson, B. (2010). Age-dependent decreases in DNA methyltransferase levels and low transmethylation micronutrient levels synergize to promote overexpression of genes implicated in autoimmunity and acute coronary syndromes. *Experimental Gerontology, 45*, 312–322.

Lord, C., Rutter, M., Goode, S., et al. (1989). Autism diagnostic observation schedule: A standardized observation of communicative and social behavior. *Journal of Autism and Developmental Disorders, 19*, 185–212.

Melnyk, S., Pogribna, M., Pogribny, I., Hine, R. J., & James, S. J. (1999). A new HPLC method for the simultaneous determination of oxidized and reduced plasma aminothiols using coulometric electrochemical detection. *The Journal Of Nutritional Biochemistry, 10*, 490–497.

Melnyk, S., Pogribna, M., Pogribny, I. P., & James, S. J. (2000). Measurement of plasma and intracellular S-adenosylmethionine and S-adenosylhomocysteine utilizing coulemetric electrochemical detection: Alteration with plasma homocysteine and pyridoxal 5'-phosphate concentrations. *Clinical Chemistry, 46*, 265–272.

Mill, J., Tang, T., Kaminsky, Z., Khare, T., Yazdanpanah, S., Bouchard, L., et al. (2008). Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. *American Journal of Human Genetics, 82*, 696–711.

Miller, A. L. (2008). The methylation, neurotransmitter, and antioxidant connections between folate and depression. *Alternative Medicine Review, 13*, 216–226.

Ming, X., Stein, T. P., Brimacombe, M., Johnson, W. G., Lambert, G. H., & Wagner, G. C. (2005). Increased excretion of a lipid peroxidation biomarker in autism. *Prostaglandins Leukotrienes and Essential Fatty Acids, 73*, 379–384.

Mohiuddin, I., Chai, H., Lin, P. H., Lumsden, A. B., Yao, Q., & Chen, C. (2006). Nitrotyrosine and chlorotyrosine: Clinical significance and biological functions in the vascular system. *Journal of Surgical Research, 133*, 143–149.

Nagarajan, R. P., Patzel, K. A., Martin, M., Yasui, D. H., Swanberg, S. E., Hertz-Picciotto, I., et al. (2008). MECP2 promoter methylation and X chromosome inactivation in autism. *Autism Research, 1*, 169–178.

Noble, M., Smith, J., Power, J., & Mayer-Proschel, M. (2003). Redox state as a central modulator of precursor cell function. *Annals of the New York Academy of Sciences, 991*, 251–271.

Pastore, A., Federici, G., Bertini, E., & Piemonte, F. (2003). Analysis of glutathione: Implication in redox and detoxification. *Clinica Chimica Acta, 333*, 19–39.

Pilger, A., & Rudiger, H. W. (2006). 8-Hydroxy-2'-deoxyguanosine as a marker of oxidative DNA damage related to occupational and environmental exposures. *International Archives of Occupational and Environmental Health, 80*, 1–15.

Reed, M. C., Thomas, R. L., Pavisic, J., James, S. J., Ulrich, C. M., & Nijhout, H. F. (2008). A mathematical model of glutathione metabolism. *Theoretical Biology & Medical Modelling, 5*, 8.

Reik, W. (2007). Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature, 447*, 425–432.

Reik, W., & Dean, W. (2001). DNA methylation and mammalian epigenetics. *Electrophoresis, 22*, 2838–2843.

Rizwana, R., & Hahn, P. J. (1999). CPG methylation reduces genomic instability. *Journal of Cell Science, 112*, 4513–4519.

Rubenstein, J. L. (2010). Three hypotheses for developmental defects that may underlie some forms of autism spectrum disorder. *Current Opinion in Neurology, 23*, 18–23.

Rubenstein, J. L., & Merzenich, M. M. (2003). Model of autism: Increased ratio of excitation/inhibition in key neural systems. *Genes, Brain, Behaviour, 2*, 255–267.

Sajdel-Sulkowska, E. M., Xu, M., & Koibuchi, N. (2009). Increase in cerebellar neurotrophin-3 and oxidative stress markers in autism. *Cerebellum, 8*, 366–372.

Samaco, R. C., Hogart, A., & LaSalle, J. M. (2005). Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. *Human Molecular Genetics, 14*, 483–492.

Schafer, F. Q., & Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine, 30*, 1191–1212.

Schanen, N. C. (2006). Epigenetics of autism spectrum disorders. *Human Molecular Genetics 15*(Spec No 2), R138–R150.

Schmutte, C., Yang, A. S., Nguyen, T. T., Beart, R. W., & Jones, P. A. (1996). Mechanisms for the involvement of DNA methylation in colon carcinogenesis. *Cancer Research, 56*, 2375–2381.

Schopler, E., Reichler, R. J., DeVellis, R. F., & Daly, K. (1980). Toward objective classification of childhood autism: Childhood Autism Rating Scale (CARS). *Journal of Autism and Developmental Disorders, 10*(1), 91–103.

Sebat, J., Lakshmi, B., Malhotra, D., Troge, J., Lese-Martin, C., Walsh, T., et al. (2007). Strong association of de novo copy number mutations with autism. *Science, 316*, 445–449.

Shigenaga, M. K., Park, J. W., Cundy, K. C., Gimeno, C. J., & Ames, B. N. (1990). In vivo oxidative DNA damage: measurement of 8-hydroxy-2'-deoxyguanosine in DNA and urine by high-performance liquid chromatography with electrochemical detection. *Methods in Enzymology, 186*, 521–530.

Small, G. W., Ercoli, L. M., Silverman, D. H., Huang, S. C., Komo, S., Bookheimer, S. Y., et al. (2000). Cerebral metabolic and cognitive decline in persons at genetic risk for Alzheimer's disease. *PNAS, 97*, 6037–6042.

Smith, M., Spence, M. A., & Flodman, P. (2009). Nuclear and mitochondrial genome defects in autisms. *Annals of the New York Academy of Sciences, 1151*, 102–132.

Smythies, J. R., Gottfries, C. G., & Regland, B. (1997). Disturbances of one-carbon metabolism in neuropsychiatric disorders: A review. *Biological Psychiatry, 41*, 230–233.

Sogut, S., Zoroglu, S. S., Ozyurt, H., Ramazan, Y. H., Ozugurlu, F., Sivasli, E., et al. (2003). Changes in nitric oxide levels and antioxidant enzyme activities may have a role in the pathophysiological mechanisms involved in autism. *Clinica Chimica Acta, 331*, 111–117.

Strous, R. D., Ritsner, M. S., Adler, S., Ratner, Y., Maayan, R., Kotler, M., et al. (2009). Improvement of aggressive behavior and quality of life impairment following S-adenosyl-methionine (SAM-e) augmentation in schizophrenia. *European Neuropsychopharmacology, 19*, 14–22.

Tchantchou, F., Graves, M., Ashline, D., Morin, A., Pimenta, A., Ortiz, D., et al. (2004). Increased transcription and activity of glutathione synthase in response to deficiencies in folate, vitamin E, and apolipoprotein E. *Journal of Neuroscience Research, 75*, 508–515.

Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W., & Pardo, C. A. (2005). Neuroglial activation and neuroinflammation in the brain of patients with autism. *Annals of Neurology, 57*, 67–81.

Vitvitsky, V., Mosharov, E., Tritt, M., Ataullakhanov, F., & Banerjee, R. (2003). Redox regulation of homocysteine-dependent glutathione synthesis. *Redox Report, 8*, 57–63.

Yan, Z., & Banerjee, R. (2010). Redox remodeling as an immunoregulatory strategy. *Biochemistry, 49*, 1059–1066.

Yan, Z., Garg, S. K., Kipnis, J., & Banerjee, R. (2009). Extracellular redox modulation by regulatory T cells. *Nature Chemical Biology, 5*, 721–723.

Yao, Y., Walsh, W. J., McGinnis, W. R., & Pratico, D. (2006). Altered vascular phenotype in autism: Correlation with oxidative stress. *Archives of Neurology, 63*, 1161–1164.

Yorbik, O., Sayal, A., Akay, C., Akbiyik, D. I., & Sohmen, T. (2002). Investigation of antioxidant enzymes in children with autistic disorder. *Prostaglandins Leukotrienes and Essential Fatty Acids, 67*, 341–343.

Zecavati, N., & Spence, S. J. (2009). Neurometabolic disorders and dysfunction in autism spectrum disorders. *Current Neurology and Neuroscience Reports, 9*, 129–136.

Zeisel, S. H. (2009). Importance of methyl donors during reproduction. *American Journal of Clinical Nutrition, 89*, 673S–677S.

Zoroglu, S. S., Armutcu, F., Ozen, S., Gurel, A., Sivasli, E., Yetkin, O., et al. (2004). Increased oxidative stress and altered activities of erythrocyte free radical scavenging enzymes in autism. *European Archives of Psychiatry and Clinical Neuroscience, 254*, 143–147.