






# A Prospective Study of Transsulfuration Biomarkers in Autistic Disorders

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**Abstract** The goal of this study was to evaluate trans-significantly ( $P < 0.001$ ) increased plasma GSSG relative sulfuration metabolites in participants diagnosed with to controls. The present observations are compatible with autism spectrum disorders (ASDs). Transsulfuration increased oxidative stress and a decreased detoxification metabolites, including: plasma reduced glutathione (GSH) capacity, particularly of mercury, in patients diagnosed with plasma oxidized glutathione (GSSG), plasma cysteine ASDs. Patients diagnosed with ASDs should be routinely plasma taurine, plasma sulfate, and plasma free sulfate tested to evaluate transsulfuration metabolites, and potential among participants diagnosed with ASDs ( $n=38$ ) in treatment protocols should be evaluated to potentially compare to age-matched neurotypical controls were to correct the transsulfuration abnormalities observed. prospectively evaluated. Testing was conducted using Vitamin Diagnostics, Inc. (CLIA-approved). Participants diagnosed with ASDs had significantly ( $P < 0.001$ ) decreased plasma reduced GSH, plasma cysteine, plasma taurine, plasma sulfate, and plasma free sulfate relative to controls. By contrast, participants diagnosed with ASDs had

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Autism spectrum disorders (ASDs) are prevalent neuro-developmental disorders that affect an estimated 1 in 150 children in the US [1]. It has been observed that ASDs are characterized by impairments in social relatedness and communication, repetitive behaviors, abnormal movement patterns, and sensory dysfunction [2]. [Further, common co-morbidity conditions often associated with ASDs include gastrointestinal disease and dysbiosis [3], autoimmune disease [4], and mental retardation [5].

In attempting to understand the underlying pathogenesis of ASDs, a considerable body of research has been conducted to evaluate potential candidate causal genes. Genetic studies, to date, have not uncovered genes of strong effect. It has recently been postulated that increasing rates of ASDs and less than 100% monozygotic concordance of ASDs support a more inclusive reframing of ASDs as a multi-system disorder with genetic influence and environmental contributors [6].

Research into the metabolic basis for ASDs has been relatively underutilized compared to other approaches.

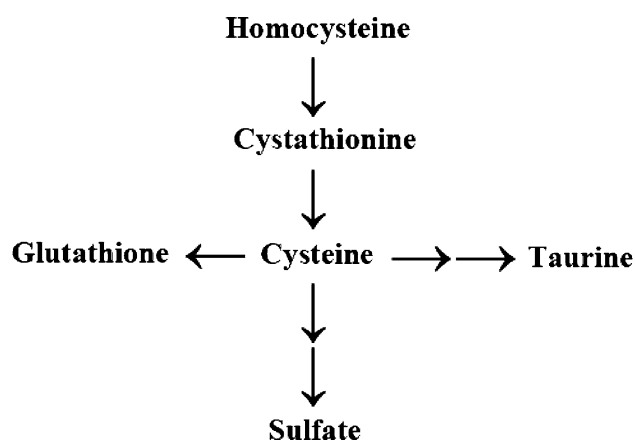


Fig. 1 A summary of the transsulfuration pathway

Several studies have recently focused on the transsulfuration pathway in ASDs. ASD children were found to have significant decreases in blood total and reduced glutathione (the major intracellular antioxidant), whereas oxidized glutathione was significantly increased in comparison with controls [7, 9]. Other researchers reported that blood levels of sulfate were significantly decreased in ASD children in comparison with controls [10]. Cysteine (the rate limiting substrate for intracellular glutathione synthesis) in ASDs was also found to be significantly decreased in plasma relative to controls [11].

A diagram of the transsulfuration pathway is presented in Fig. 1. The transsulfuration pathway starts with homocysteine, which can either be remethylated to methionine or irreversibly removed from the methionine cycle by CBS, which permanently removes homocysteine from the methionine cycle and initiates the transsulfuration pathway for the synthesis of cysteine, glutathione, sulfate, and taurine as indicated in Fig. 1 [11].

The present study was undertaken to confirm and extend previous observations in patients diagnosed with ASDs by examining a different cohort of children diagnosed with ASDs using routine, clinically available lab testing. The purpose of the present study was to further evaluate an entire metabolic pathway (i.e. the transsulfuration pathway), as opposed to isolated single gene products, to provide a greater mechanistic insight into disease pathology, so that new options for targeted treatment strategies may be further explored.

#### Experimental Procedure

The study was conducted at the Autism Treatment Center (Dallas, Texas). Phlebotomy took place at Medical Center Plano, Outpatient Phlebotomy (Plano, Texas).

The study protocol received Institutional Review Board (IRB) approval from Liberty IRB, Inc. (Deland, Florida). All parents signed a consent and Health Insurance Portability and Accountability Act (HIPAA) form and all received a copy. Children were in the presence of one or both parents at all times during the study.

#### Participants

The present study looked at qualifying participants ( $n = 38$ ) who were prospectively recruited from the community of the Dallas/Fort Worth, Texas area. All of the children had a diagnosis of autism or pervasive developmental disorder (PDD). Children included in the present study were between 2 and 16 years of age and had an initial Childhood Autism Rating Scale (CARS) score  $\geq 30$ . A

child with a CARS score  $\geq 30$  is considered to have autism [12]. This study excluded children who had a history of significant medical issues (e.g. fragile X disorder, tuberous sclerosis, phenylketonuria (PKU), Lesch-Nyhan syndrome, fetal alcohol syndrome, or history of maternal illicit drug use). Clinical Evaluation regarding demographics, formal diagnosis, age at diagnosis, age of apparent onset, information regarding delay or depression, any current medical issues, medications, and allergies on each child. A baseline CARS evaluation was performed by Dr. Kern, who was trained in the use of CARS, and has 12 years experience in using the CARS to evaluate more than 300 persons with an ASD diagnosis. Dr. Kern interviewed the parents and observed each child. Table 1 summarizes the pertinent demographics of the participants included in the present study.

Following the intake evaluation, each participant in the present study had blood samples collected. The laboratory specimens were all collected in the morning following an overnight fast. Specimens were immediately taken to and processed at LabCorp in Medical City Hospital (Dallas, Texas) and then shipped overnight to Vitamin Diagnostics, Inc. (Cliffwood Beach, New Jersey). The lab used in the present study was blinded and received no information regarding the clinical status of the participants examined or their CARS scores prior to their testing of each sample.

Participants were tested for the following at Vitamin Diagnostics (all CLIA-approved): transsulfuration metabolites including plasma cysteine, plasma taurine, plasma reduced glutathione, plasma oxidized glutathione, plasma free sulfate, and plasma total sulfate.

Table 1 A summary of the participants with ASD included in the present study

Descriptive information	
Sex/age	
Male/female (ratio)	34/4 (8.5:1)
Mean age in years ± Std (range)	6.0 ± 2.6 (2-13)
Race (n)	
Caucasian	71% (27)
Hispanic	7.9% (3)
Black	7.9% (3)
Asian	7.9% (3)
Mixed	5.3% (2)
Autistic disorder characteristics	
Mean CARS score ± Std (range)	39 ± 6.3 (30-51)
Regressive (n)	65.8% (25)
Non-regressive (n)	34.2% (13)
Autism (n)	73.7% (28)
Autism spectrum disorders (n)	26.3% (10)
Previous treatments	
Supplements (n)	42.1% (16)
Chelation (n)	0% (0)
Supplements + chelation (n)	18.9% (7)

Std = standard deviation. All participants examined in the present study were living in the state of Texas

<sup>a</sup> Includes participants that had a regressive event in development at any time following birth

<sup>b</sup> Autism spectrum disorders include participants diagnosed with pervasive developmental disorder not otherwise specified (PDD-NOS) and Asperger's disorder

Lab Methods

Blood Transsulfuration Metabolites

Plasma oxidized and reduced glutathione samples were collected immediately after venipuncture by adding collected plasma to a preservative solution in order to stop any reaction which might change the ratio of oxidized to reduced glutathione. Both reduced and oxidized glutathione were measured. Liquid chromatography followed by tandem mass spectrometry was used. Total plasma cysteine and plasma taurine samples were collected immediately after venipuncture by adding collected plasma to a preservative solution. The stabilized plasma was used to quantify total plasma cysteine by a homogenous enzymatic colorimetric assay [14] and plasma taurine was determined by HPLC/fluorescence technique [15]. Total plasma sulfate per g protein and plasma free sulfate per g protein samples were collected immediately after venipuncture by adding collected plasma to a preservative solution. Sulfate was determined using the procedure of Chattaraji and Das [16]. A Shimadzu Model 646 atomic

absorption spectrometer was used under the following conditions: copper lamp current 7 mA; wavelength 325 nm; slit-width, 0.38 nm; acetylene flow plate, 1.5<sup>3</sup>dm min; and air flow plate, 10.0 dm<sup>3</sup>min. Free inorganic sulphate was measured by negative electrospray ionization tandem mass spectrometry [17]. To the sample <sup>34</sup>S-labeled sodium sulphate was added as an internal standard. The sample was deproteinized with methanol and bicarbonate anions titrated with dilute acetic acid to pH 7.0. The tandem mass spectrometer was used in neutral loss mode to detect HSQ<sup>-</sup> ions. To determine the quantity of protein, 100 µl of a 0.15% solution of sodium deoxycholate was added to a 1.8 ml sample of plasma. After 10 min at 4 °C, 100 µl of trichloroacetic acid (6N) was added. The mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. The precipitate was solubilized with 50 µl of sodium dodecyl-sulfate (2.5%) in 0.01N NaOH. When the precipitate was completely dissolved, 45 µl of HCl (0.01N) was added and assayed for protein by the method of Watanabe et al. [18]. The recovery of protein was 87 ± 5% (n = 16).

Controls

The transsulfuration metabolites of plasma reduced glutathione, plasma oxidized glutathione, plasma sulfate, plasma free sulfate, plasma taurine measured at Vitamin Diagnostics among participants with an ASD diagnosis were compared to prospective samples collected by testing neurotypical boys and girls between 2-16 years of age by the lab (n = 25). Significant sex-specific differences were not observed among the neurotypical boys and girls for transsulfuration metabolites tested, so control samples were pooled across sex.

Statistical Analyses

The current study used the statistical package contained in StatsDirect (Version 2.4.2). For each participant, his or her transsulfuration levels were evaluated in relation to the mean level from neurotypical controls using the unpaired parametric t-test statistic. Additionally, for each participant, his or her transsulfuration (Vitamin Diagnostics) levels were evaluated in relation to the mean level from neurotypical controls, so as to convert each participant's measured test values into a percent of the mean value of neurotypical controls (participant's laboratory value/mean level from neurotypical controls] × 100 = percent of the pertinent mean). For each metabolite examined, the individual results were then averaged to compute an overall average percent of the pertinent means, and the standard error for each characteristic was calculated. The null hypothesis was that there should be no difference in means among the participants with an ASD and neurotypical controls for each metabolite

Table 2 An assessment of transsulfuration metabolites among the participants diagnosed with an ASD in comparison to neurotypical controls

Lab test	Mean $\pm$ Std (% of pertinent mean $\pm$ SEM)	Mean $\pm$ Std neurotypical controls (n) <sup>f</sup>	P-value <sup>b</sup>	% [ Controls upper limit <sup>f</sup> (n)	% \ Controls lower limit <sup>c</sup> (n)
Plasma cysteine $\mu$ (mol/l)	17.8 $\pm$ 8.3 (77 $\pm$ 5.8)	23.2 $\pm$ 4.2 (64)	\ 0.001	5.3 (2)	36.8 (14)
Plasma reduced glutathione $\mu$ (mol/l)	3.14 $\pm$ 0.56 (75 $\pm$ 2.2)	4.2 $\pm$ 0.72 (120)	\ 0.0001	0 (0)	26.3 (10)
Plasma oxidized glutathione (nmol/l)	0.48 $\pm$ 0.16 (137 $\pm$ 7.4)	0.35 $\pm$ 0.05 (120)	\ 0.001	60.5 (23)	13.2 (5)
Plasma taurine $\mu$ (mol/l)	48.6 $\pm$ 14.0 (50 $\pm$ 2.3)	97.5 $\pm$ 8.8 (27)	\ 0.0001	0 (0)	100 (38)
Plasma total sulfate $\mu$ (mol/g P)	934 $\pm$ 252 (48 $\pm$ 2.1)	1,930 $\pm$ 184 (82)	\ 0.0001	0 (0)	100 (38)
Plasma free sulfate $\mu$ (mol/g P)	1.37 $\pm$ 0.48 (33 $\pm$ 1.9)	4.1 $\pm$ 0.46 (67)	\ 0.0001	0 (0)	100 (38)

Std = standard deviation; SEM = standard error of the mean

<sup>a</sup> Prospective samples collected by testing neurotypical boys and girls, from the United States, between 2–16 years of age by the lab

<sup>b</sup> The unpaired-test statistic was utilized

<sup>c</sup> Mean $\pm$  (2.9 standard deviation)

examined. For all the statistical tests in the present study, particular concern. Glutathione is a tripeptide of cysteine, two-tailed P-value  $\leq 0.05$  was considered statistically significant.

## Results

Table 2 summarizes an assessment of transsulfuration metabolites among the participants with ASD in comparison to the neurotypical controls examined in the present study. Overall, it was observed that the participants with ASD had significantly decreased levels of plasma cysteine, plasma reduced glutathione, plasma taurine, plasma total sulfate, and plasma free sulfate. The abnormalities were greatest in free sulfate (ASD mean was 33% of control mean), followed by total sulfate and taurine (ASD means were 48 and 50% of control means, respectively), with less difference in reduced glutathione and cysteine (ASD means were 75 and 77% of control means, respectively).

By contrast, participants with ASD had significantly increased plasma oxidized glutathione (ASD mean was 137% of control mean). Additionally, no significant differences were observed for the transsulfuration metabolites when comparing participants with a prior history of supplementation and/or chelation therapy (data not shown). almost 40% of the participants diagnosed with ASDs relative to controls. It is important to note that cysteine is a conditionally essential amino acid that is dependent on adequate methionine status; thus, a decrease in methionine precursor levels effectively increases the requirement for reformed cysteine. The significant decrease in plasma abnormalities in the biochemical markers in the transsulfuration pathway among participants diagnosed with ASDs, oxidized glutathione observed among the study participants with ASDs suggest that precursor availability is insufficient to maintain glutathione levels and normal redox homeostasis. Consistent with low plasma reduced and total glutathione levels and increased oxidative stress, autistic

## Discussion

The overall results of the present study showed significant abnormalities in the biochemical markers in the transsulfuration pathway among participants diagnosed with ASDs, oxidized glutathione observed among the study participants with ASDs suggest that precursor availability is insufficient to maintain glutathione levels and normal redox homeostasis. Consistent with low plasma reduced and total glutathione levels and increased oxidative stress, autistic

The significant decrease in plasma reduced glutathione and increased oxidized glutathione among the participants diagnosed with ASDs relative to neurotypical controls is of glutathione levels and increased oxidative stress, autistic

children would be expected to have difficulty resisting brain that have received attention from brain studies in infection, resolving inflammation, and detoxifying environmental contaminants. Indeed, patients diagnosed with ASDs were reported to suffer from recurrent infections [28], neuroinflammation [29], gastrointestinal inflammation [30, 31], and impaired antioxidant and detoxification capacity [32, 34].

Research in rats has also shown gender differences in detoxification, with females excreting significantly higher levels of mercury than males [45, 46]. Other researchers found that males are more dependent on sulfotransferase activity for the removal of xenobiotics [47]. In addition, availability and mercury excretion has been found [35].

Bile is the main route of elimination for many metals, and a step in the transsulfuration pathway, is down-regulated by the rate of secretion of methyl and inorganic mercury into testosterone in human cells. This results in a significant decrease in flux through the transsulfuration pathway and rates soon after weaning. These changes closely parallel lower intracellular glutathione levels [48]. Furthermore, it was observed that when reduced glutathione secretion into bile was completely inhibited, induced significant increases in neurotoxic effects in males without changing hepatic levels of reduced glutathione when compared to females [49]. Overall, these observations may be particularly important to patients diagnosed with ASDs, since the male/female ratio in ASDs is at least 3:1 [16], and since researchers have reported significant increased glutathione in patients diagnosed with ASDs [30]. Because sulfate and glutathione are essential for effective detoxification, the effects of a lack of availability of

free sulfate and reduced glutathione on detoxification are far-reaching. Exposure to toxins in children with compromised detoxification capability has an even greater potential to disrupt critical developmental processes and result in developmental neurotoxicity [51].

Lack of availability of free sulfate and reduced glutathione may be only one part of the issue. Examination of the effects of heavy metals reveals that the presence of heavy metals, e.g., mercury, can disrupt the very processes needed to excrete the metals. Evidence shows that metal ions disrupt methionine synthetase which then, results in the inhibition of glutathione production [52]. In addition, the presence of metals causes oxidative stress, and since glutathione has the dual function of both reducing oxidative stress and detoxifying heavy metals, glutathione may be become rapidly depleted as a result of demand. The overall importance of these phenomena in relation to individuals diagnosed with ASDs, as observed in the present study, is that plasma cysteine, plasma sulfate, plasma taurine, and plasma reduced glutathione were all significantly decreased in participants diagnosed with ASDs, whereas by contrast, plasma oxidized glutathione was significantly increased in participants diagnosed with ASDs. These findings are in agreement with observations made by previous researchers [10]. Like the current study, these previous studies have shown that, relative to the controls, individuals with ASDs had significant reductions in blood levels of glutathione, cysteine, and sulfate, whereas by contrast, plasma oxidized glutathione was significantly increased.

The brain has many sulfate transporters. Sulfate transporters are expressed most highly in the cerebellum and hippocampus, suggesting that in these locations, important processes needing sulfate regulation are taking place [42]. Additionally, cysteine dioxygenase (CDO), the rate limiting enzyme of cysteine oxidation, is strongly expressed in the Purkinje neurons of the cerebellum and in neurons in the hippocampus [43], probably because the supply of sulfate is so vital to the function in that region. The hippocampus and the cerebellum are the two places in the

## Strengths and Limitations

The present study has number of potential strengths that help to support the observations made. First, the design of the present study, as a prospective, blinded study, helps to minimize the chance for selection bias of study participants. In addition, the blinded nature of the study ensured that biasing factors regarding clinical or lab assessments of individual participants were minimized because neither cohort group was aware of the other's results.

Second, since the present study was conducted at the ATC, a non-biomedical intervention center, the patients examined in the present study were a priori not skewed toward those seeking biomedical interventions at a physician's office. The participants examined in the present study were selected from community contacts.

Third, and most importantly, the consistency and specificity of the results observed were strengths of the present study. It was observed that each transsulfuration metabolite examined, with the exception of plasma oxidized glutathione, was significantly decreased relative to the neurotypical controls.

Finally, since two-tailed *P*-values were used, and the directions of the significant effects observed were in the biologically plausible directions, the mere chance occurrence of observing the results found in the present study were minimal. Furthermore, since 20 total statistical tests were generated in the present study, a two-tailed *P*-value *B*0.05 was considered significant, and all of the *P*-values calculated were *B*0.01, it is reasonable to conclude that the results observed were not due to statistical chance.

In considering the potential limitations of the present study, the number of study participants was of moderate size. Despite this potential limitation of the present study, it was observed that there were consistent statistical effects observed. It would be worthwhile to evaluate the consistency of the results observed here with those in different and expanded cohorts of individuals diagnosed with ASDs. Additionally, in the present study, data was not evaluated concerning other biomarkers of oxidative stress or heavy metal toxicity present in the study participants examined. It would be of value in future studies to examine if there was a potential correlation between other biomarkers of oxidative stress or heavy metal toxicity and transsulfuration biomarkers among individuals diagnosed with ASDs.

## Conclusion

The present study is the first prospective study conducted to evaluate transsulfuration metabolites in a cohort of patients diagnosed with ASDs using routinely available clinical lab testing. For the study participants examined,

this study found that they had significant evidence of decreased plasma levels of the transsulfuration metabolites of cysteine, taurine, sulfate, free sulfate, and reduced glutathione. By contrast, it was also found that they had significant evidence of increased levels of the transsulfuration metabolite of plasma oxidized glutathione. We recommend that future studies should focus on further evaluating transsulfuration metabolites in an expanded cohort of individuals diagnosed with ASDs, and potential treatment protocols be evaluated to potentially correct the transsulfuration abnormalities observed in the present study. Additionally, we suggest that future studies of individuals diagnosed with ASDs should examine the potential correlation between biomarkers of oxidative stress or heavy metal toxicity and transsulfuration biomarkers. Finally, we recommend, since the lab testing employed in the present study for examining transsulfuration metabolites is clinically available, relatively inexpensive, and relatively noninvasive, that patients diagnosed with ASDs be routinely tested to evaluate them.

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