Biomarkers of environmental toxicity and susceptibility in autism ☆

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ABSTRACT

Autism spectrum disorders (ASDs) may result from a combination of genetic/biochemical susceptibilities in the form of a reduced ability to excrete mercury and/or increased environmental exposure at key developmental times. Urinary porphyrins and transsulfuration metabolites in participants diagnosed with an ASD were examined. A prospective, blinded study was undertaken to evaluate a cohort of 28 participants with an ASD diagnosis for Childhood Autism Rating Scale (CARS) scores, urinary porphyrins, and transsulfuration metabolites. Testing was conducted using Vitamin Diagnostics, Inc. (CLIA-approved) and Laboratoire Philippe Auguste (ISO-approved). Participants with severe ASDs had significantly increased mercury intoxication-associated urinary porphyrins (pentacarboxyporphyrin, precoproporphyrin, and coproporphyrin) in comparison to participants with mild ASDs, whereas other urinary porphyrins were similar in both groups. Significantly decreased plasma levels of reduced glutathione (GSH), cysteine, and sulfate were observed among study participants relative to controls. In contrast, study participants had significantly increased plasma oxidized glutathione (GSSG) relative to controls. Mercury intoxication-associated urinary porphyrins were significantly correlated with increasing CARS scores and GSSG levels, whereas other urinary porphyrins did not show these relationships. The urinary porphyrin and CARS score correlations observed among study participants suggest that mercury intoxication is significantly associated with autistic symptoms. The transsulfuration abnormalities observed among study participants indicate that mercury intoxication was associated with increased oxidative stress and decreased detoxification capacity.

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1. Introduction

Autism spectrum disorders (ASDs) are prevalent neurodevelopmental disorders that, based on a recent survey, affect not less than 1 in 150 children born in the US during the early 1990s [1]. ASD diagnoses 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 an ASD diagnosis include gastrointestinal disease and dysbiosis [3], autoimmune disease [4], and mental retardation [5].

In attempting to understand the underlying pathogenesis in those with an ASD diagnosis, 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 and less than 100% monozygotic concordance 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. In considering potential environmental contributors to ASDs, some studies have reported that exposure to mercury can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with autistic disorders, and that these similarities extend to neuroanatomy, neurotransmitters, and biochemistry [7–9]. In addition, investigators from the US National Institute of Environmental Health Sciences [10] and the National

Institute for Occupation Safety and Health of the Centers for Disease Control and Prevention [11] have described a role for mercury exposure in the pathogenesis of autism. Mercury poisoning has also sometimes been presumptively diagnosed as autism of unknown etiology until mercury poisoning has been uncovered [12]. Further, investigators reported on the effects of mercury on neuronal development: "…mercury exposure altered cell number and cell division; these impacts have been postulated as modes of action for the observed adverse effects in neuronal development. The potential implications of such observations are evident when evaluated in context with research showing that altered cell proliferation and focal neuropathologic effects have been linked with specific neurobehavioral deficits (e.g., autism)" [13]. Finally, the Collaborative on Health and the Environment's Learning and Developmental Disabilities recently published a consensus statement reporting that there is no doubt mercury exposure may produce autism spectrum disorders [14].

It may be hypothesized that autism results from a combination of genetic and biochemical susceptibilities in the form of a reduced ability to excrete mercury and/or increased environmental exposure at key times in development. This would mean that individuals exposed to relatively high mercury could be affected even if their bodies were innately efficient eliminators. In contrast, only if an exposed fetus or infant also had genetic and/or biochemical susceptibilities, which decrease one's ability to remove mercury, would a lesser level of mercury exposure lead to problems.

In order to clinically examine evidence for the above hypothesis, it is important to analyze biomarkers for mercury susceptibility and toxicity in patients diagnosed with an ASD. Namely, it was previously demonstrated that the transsulfuration pathway products of glutathione [15] and sulfate [16] were related to mercury excretion rates, and that the heme synthesis pathway products of urinary porphyrins can provide specific profiles that reflect mercury toxicity [17]. Fig. 1 summarizes the biochemical steps involved in the transsulfuration and heme synthesis pathways.

The purpose of the present prospective, blinded study was to evaluate potential biomarkers using clinically available lab testing for evidence of mercury susceptibility and toxicity in the transsulfuration and porphyrin pathways in a cohort of participants diagnosed with ASDs.

2. Materials and methods

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.

2.1. Participants

The present study examined consecutive qualifying participants ($n=28$) who were prospectively recruited from the community of Dallas/Fort Worth. All of the children selected had a diagnosis of autism or pervasive developmental disorder (PDD) and had not previously undergone chelation therapy. Children included in the present study were between 2 and 16 years of age and had an initial Childhood Autism Rating Scale (CARS) score [18] ≥30. A child with a CARS score ≥30 is considered to have autism. This study excluded children who had a history of Fragile X disorder, tuberous sclerosis, phenylketonuria (PKU), Lesch–Nyhan syndrome, fetal alcohol syndrome, or history of maternal illicit drug use.
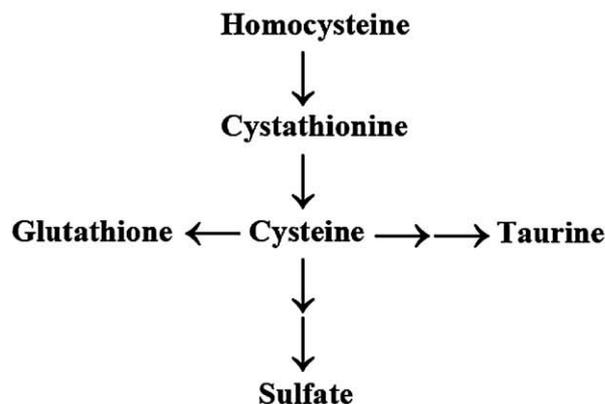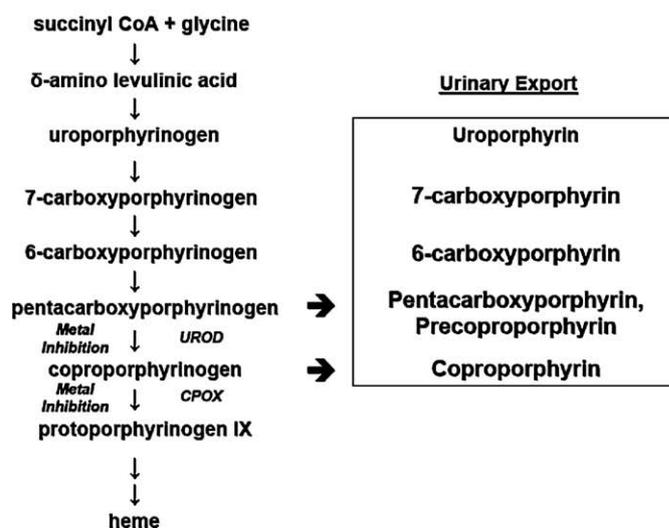

Fig. 1. A summary of the transsulfuration and heme pathways. Porphyrinogens appear in urine as porphyrin derivatives (right). Mercury can cause increased urinary 5cxP, prcP, and cP by inhibiting uroporphyrinogen decarboxylase (UROD) and/or coproporphyrinogen oxidase (CPOX); urinary uroporphyrin is not reported to alter with inhibition of these enzymatic steps.

2.2. Clinical evaluation

As a baseline, information was obtained regarding demographics, formal diagnosis, age at diagnosis, age of apparent onset, information regarding delay or regression, any current medical issues, medications, and allergies on each child. A baseline CARS evaluation was performed by Dr. Kern who interviewed the parents and observed each child. Dr. Kern is 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. Table 1 summarizes the pertinent demographics of the participants included in the present study.

2.3. Lab evaluation

Following the intake evaluation, each participant in the present study had blood and urine samples collected. The laboratory specimens were all collected in the morning following an overnight fast. Urine samples were collected from participants as first morning urine samples. Specimens were immediately taken to and processed at LabCorp in Medical City Hospital (Dallas, Texas) and then shipped to the following labs: (1) Vitamin Diagnostics, Inc. (Cliffwood Beach, New

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

Descriptive information	Overall (n=28)	Mild ASD[a] (n=14)	Severe ASD[b] (n=14)
Sex/age			
Male/female (ratio)	23/5 (4.6:1)	12/2 (6:1)	11/3 (3.7:1)
Mean age in years±Std (range)	5.8±2.7 (2–13)	6.2±3.1 (3–13)	5.4±2.2 (2–9)
Race (n)			
Caucasian	71.4% (20)	78.6% (11)	64.3% (9)
Minorities[c]	28.6% (8)	21.4% (3)	35.7% (5)
Autistic disorder characteristics			
Mean CARS Score±Std (range)	38.2±5.7 (30–51)	33.6±3.1 (30–38.5)	42.8±3.6 (38.5–51)
Regressive (n)[d]	57.1% (16)	57.1% (8)	57.1% (8)
Non-regressive (n)	42.9 (12)	42.9% (6)	42.9% (6)
Autism (n)	71.4% (20)	64.3% (9)	78.6% (11)
Autism spectrum disorders (n)[e]	28.6% (8)	35.7% (5)	21.4% (3)
Previous treatments			
Supplements (n)	33.3% (9)	21.4% (3)	42.9% (6)

Std = standard deviation.
All participants examined in the present study were living in the state of Texas and had not previously received chelation therapy.

[a] Mild ASD is defined as any study participant with a CARS score less than the overall study participant median (CARS score ≤38.5).

[b] Severe ASD is defined as any study participant with a CARS score greater than the overall study participant median (CARS score ≥38.5).

[c] Includes participants of Hispanic, Black, Asian, or Mixed Ancestry.

[d] Includes participants that had a regressive event in development at any time following birth.

[e] Autism spectrum disorders include participants diagnosed with pervasive developmental disorder—not otherwise specified (PDD-NOS) and Asperger's disorder.

Jersey); and (2) Laboratoire Philipe Auguste (Paris, France). The laboratories used in the present study were 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: (1) Tests at Vitamin Diagnostics (all CLIA-approved): transsulfuration metabolites including plasma cysteine, plasma reduced glutathione, plasma oxidized glutathione, and plasma total sulfate; and (2) Tests at Laboratoire Philippe Auguste, Paris, France (all ISO-approved) urinary porphyrin testing including uroporphyrin (uP), heptacarboxyporphyrin (7cxP), hexacarboxyporphyrin (6cxP), pentacarboxyporphyrin (5cxP), preco-proporphyrin (prcP), and coproporphyrin (cP).

2.4. Lab methods

2.4.1. Urinary porphyrin metabolites

Analyses of urinary porphyrins, blinded for the diagnoses of the study participants, were conducted. Study participants' first morning urine samples (10 mL) were collected in vacuette (Greiner Bio-One, Les Ulis, France), maintained in the dark at ambient temperature, and shipped to the Laboratoire Philippe Auguste by standard air mail. Upon arrival at the Laboratoire Philippe Auguste, the specimens were frozen (−20 °C) until analysis. Porphyrins were determined by HPLC Spectrofluorimetric technique: after centrifugation (3000 ×g, 5 min), 500 µL urine were acidified with 50 µL of aqueous hydrochloric acid (37% w/v), recentrifuged, and 20 µL of the resulting sample injected into an Econosphere column (C-18, 5 µm particle size, 250×4.6 mm, Alltech, Templemars, France). Elution from the column was effected using a gradient (Phase A: 50 mmol KH_2PO_4, pH adjusted to 3.5 with acetic acid [CH_3COOH]; Phase B: high-purity methanol [CH_3OH]). Eluant flow was 1 mL/min and the following A:B gradient: 0 min A/B 50:50, 2 min 35:65, 5 min 15:85, 15 min 1:99, and 28 min 50:50 was applied. Fluorescence detection and measurement (excitation 405 nm, emission 618 nm) were used for the porphyrins in system that had dual in-line detection capability (UV model 310, Fluorescence model

363 both from Varian, Les Ulis, France). The nominal porphyrin retention times (in minutes) were: 7.3, 8.6, 10.2, 11.7, 12.7 and 13.9 for uP, 7cxP, 6cxP, 5cxP, prcP, and cP, respectively. The I and III isomers of uP and cP were not separated. The fluorescence responses from the samples were quantified against the corresponding responses from a mixed porphyrin reference sample (Porphyrin Products, Logan, Utah). The urinary creatinine level in each sample was measured by a spectrophotometric assay (Vitros, Ortho-Clinical Diagnostics, Johnson and Johnson). The porphyrin values reported were expressed in nanomoles per g of creatinine measured [19].

2.4.2. 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 [20].

Total plasma cysteine 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 [21]. Cysteine was measured in the non-protein fraction of the plasma. This method was designed to measure only circulating free cysteine and not total cysteines which may come from cystines and other non-protein sulfhydryl groups in blood. To the plasma at 4 °C, equal volume of acetone at −10 °C was added. After overnight precipitation at 4 °C the sample was centrifuged at 4 °C for 15 min at 4000 rpm. The supernatant was separated and placed under nitrogen to remove the acetone and then lyophilized. The residue was dissolved in 1 mL of buffer containing 0.05 M citrate buffer, 1% glycerol, 1% ascorbic acid

Table 2
An assessment of urinary porphyrin and creatinine levels among the participants with a mild ASD[a] in comparison to participants with a severe ASD[b]

Lab test	Mild ASD cases (n=14) mean±Std [median]	Severe ASD cases (n=14) mean±Std [median]	p-value[c]
Nanomoles/gram creatinine			
Uroporphyrins I and III	25.52±7.77 [22]	23.24±6.68 [23]	NS
Heptacarboxyporphyrin	4.41±1.44 [4.9]	4.92±1.77 [4.2]	NS
Hexacarboxyporphyrin	0.91±0.6 [0.76]	0.91±0.52 [0.96]	NS
Pentacarboxyporphyrin	4.92±2.79 [4.4]	6.0±1.58 [6.2]	<0.05
Precoproporphyrin	17.3±8.9 [15]	24.05±7.28 [23]	<0.05
Coproporphyrins I and III	237±90 [196.5]	285±92.1 [257]	NS
Ratios			
Heptacarboxyporphyrin/ uroporphyrins I and III	0.18±0.05 [0.16]	0.22±0.09 [0.20]	NS
Hexacarboxyporphyrin/ uroporphyrins I and III	0.04±0.03 [0.03]	0.04±0.02 [0.04]	NS
Pentacarboxyporphyrin/ uroporphyrins I and III	0.19±0.06 [0.185]	0.27±0.08 [0.28]	<0.005
Precoprophyrin/uroporphyrins I and III	0.68±0.28 [0.665]	1.09±0.36[1.1]	<0.005
Coproporphyrins I and III/ uroporphyrins I & and III	9.54±2.97 [9.7]	12.64±3.47 [12.54]	<0.05
(Precoproporphyrin+ pentacarboxyporphyrin)/ (uroporphyrins I & and III+ heptacarboxyporphyrin)	0.73±0.26 [0.7]	1.1±0.29 [1.12]	<0.005
Creatinine (mg/L)	977±427 [867.5]	925±491 [954]	NS

Std = standard deviation.
NS = not significant.

[a] Mild ASD is defined as any study participant with a CARS score less than the overall study participant median.

[b] Severe ASD is defined as any study participant with a CARS score greater than the overall study participant median.

[c] The unpaired non-parametric Mann Whitney U test statistic was utilized (two-tailed).

Total CARS Score
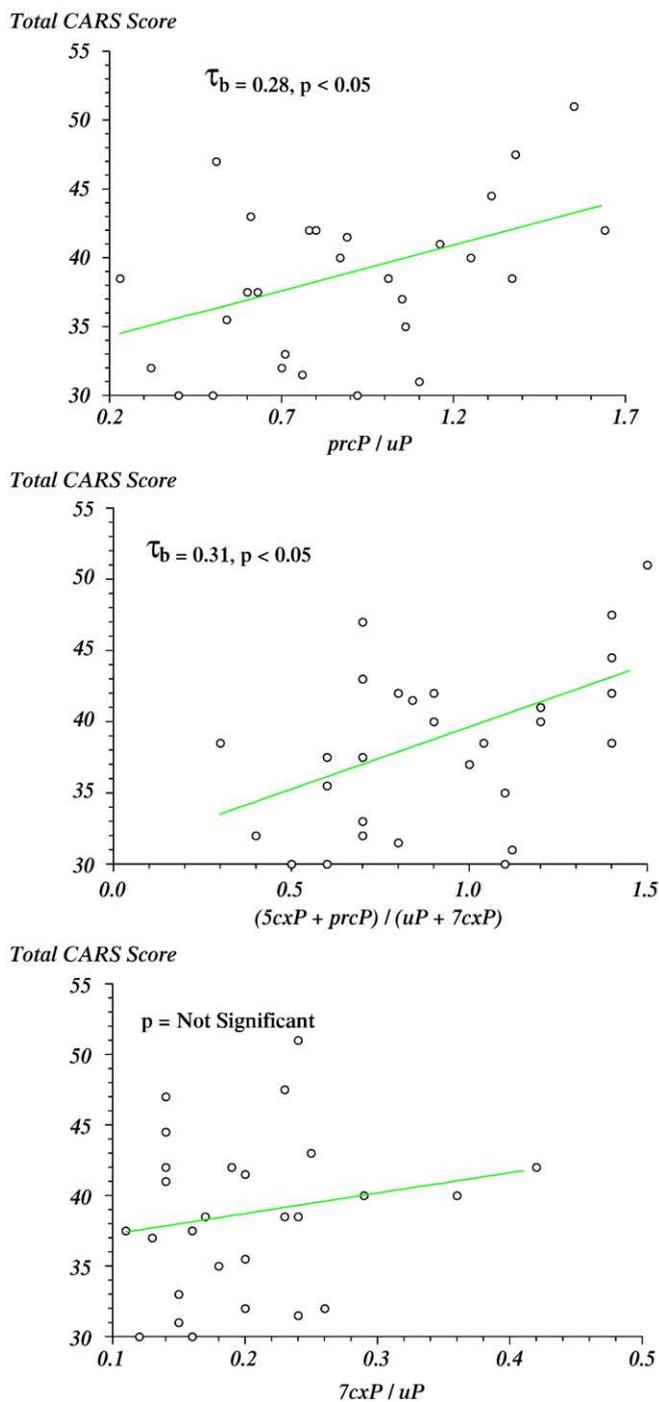

Total CARS Score

Total CARS Score

Fig. 2. A summary of the correlation (non-parametric linear regression test statistic was utilized (two-tailed)) between urinary porphyrins and CARS scores.

and 0.5% sodium metabisulphite; pH 5.5 at 4 °C in order to maintain low redox potential under reducing condition. Cysteine was measured spectrophotometrically without the addition of dithiothreitol [21]. This method was validated by two independent methods including: (1) HPLC method with dual electrochemical detection [22] and (2) Ellman's test [23]. Deming regression analysis with both methods yielded the following results with HPLC ($y = 1.0033x - 0.4278$, $R^2 = 0.96$, $n = 52$) and Ellman's test ($y = 1.0102x - 0.6327$, $R^2 = 0.93$, $n = 52$).

Samples for total plasma sulfate per g protein were prepared immediately after venipuncture by adding collected plasma to a preservative solution. Sulfate in these samples was determined using the procedure of Chattaraji and Das [24]. 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 dm³/min; and airflow plate, 10.0 dm³/min. Free inorganic sulphate was measured by negative electrospray ionization tandem mass spectrometry [25]. First, ^{34}S-labeled sodium sulphate was added to the sample as an internal standard. The resultant 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 HSO_4^- 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 tricholoacetic acid (6 N) 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 deodecylsulfate (2.5%) in 0.01 N NaOH. When the precipitate was completely dissolved, 450 μL of HCl (0.01 N) was added and assayed for protein by the method of Watanabe et al. [26]. The recovery of protein was 87 ± 5% ($n = 16$).

2.5. Controls

The transsulfuration metabolites (Vitamin Diagnostics) measured in the samples provided by the participants with an ASD diagnosis were compared to prospective samples collected by testing neurotypical boys and girls between the ages of 2–16 years of age by the lab ($n > 25$). Significant sex-specific differences were not observed among the neurotypical boys and girls for the transsulfuration metabolites tested, so control samples were pooled across sex.

2.6. Statistical analyses

The current study used the statistical package contained in StatsDirect (Version 2.4.2). Urinary porphyrins between participants with mild ASDs (CARS score < median overall score) in comparison with severe ASDs (CARS score > median overall score) were evaluated utilizing the unpaired non-parametric Mann Whitney U test statistic. The null hypothesis stated that there should be no difference between the median for each urinary porphyrin between participants with mild and severe ASDs. The non-parametric linear regression test statistic was utilized to evaluate the relationship between urinary porphyrin levels and CARS scores for the study participants. The null hypothesis stated that the slope of the line would be equal to zero for the relationship between urinary porphyrin levels and CARS scores. Transsulfuration levels were evaluated in relation to the mean level from neurotypical controls using the parametric t-test statistic. The null hypothesis stated that there should be no difference in means among the participants with an ASD and neurotypical controls for each metabolite examined. Finally, plasma oxidized glutathione levels were evaluated in relationship to urinary porphyrin levels by examining the overall plasma oxidized glutathione levels in those with low (urinary porphyrin level < overall median level) in comparison to high (urinary porphyrin level > overall median level) urinary

Table 3

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

Lab test	Mean ± Std overall ASD participants ($n = 28$)	Mean ± Std controls[a] (n)	p-value[b]
Plasma cysteine (μmol/L)	17.8 ± 9.5	23.2 ± 4.2 (64)	< 0.01
Plasma reduced glutathione (μmol/L)	3.1 ± 0.53	4.2 ± 0.72 (120)	< 0.0001
Plasma oxidized glutathione (nmol/L)	0.46 ± 0.16	0.35 ± 0.05 (120)	< 0.005
Plasma total sulfate (μmol/g P)	924 ± 245	1930 ± 184 (82)	< 0.0001

Std = standard deviation.
[a] Prospective samples collected by testing neurotypical boys and girls between the ages of 2–16 years of age by the lab.
[b] The unpaired parametric t-test statistic was utilized (two-tailed).

porphyrin levels for each type examined in the present study utilizing the unpaired non-parametric Mann Whitney U test statistic. The null hypothesis stated that there should be no difference between the median for plasma oxidized glutathione between participants with low in comparison to high urinary porphyrin levels for each type examined. For all the statistical tests in the present study, a two-tailed p value ≤ 0.05 was considered statistically significant.

3. Results

Table 2 lists the urinary porphyrin and creatinine levels among the participants with mild ASDs in comparison to participants with severe ASDs, defined as a CARS score below or above the median (38.5), respectively. It was observed that there were significant increases in the means for 5cxP/g of creatinine (1.2-fold) and prcP/g of creatinine (1.4-fold) in participants with severe ASDs in comparison to mild ASDs. In contrast, no significant differences were observed in the other urinary porphyrin and creatinine levels measured among participants with severe ASDs in comparison to mild ASDs. In addition, it was also observed that there were significant increases in the mean ratios for 5cxP/uP (1.4-fold), prcP/uP (1.6-fold), cP/uP (1.3-fold) and (5cxP+prcP)/(7cxP+uP) (1.5-fold) among participants with severe ASDs in comparison to mild ASDs. In contrast, no significant differences were observed in the ratios for the other urinary porphyrins measured among participants with severe ASDs in comparison to mild ASDs. It was also observed that 43% of participants with severe ASDs had prcP/uP ratios higher than the mean+2 standard deviations of participants with mild ASDs.

Fig. 2 shows the correlation between CARS scores and specific urinary porphyrin levels among the participants examined. It was found that there were significant correlations were between the CARS scores and prcP/uP ratios (τ_b=0.28, p<0.05) and between the CARS scores and [(5cxP+prcP)/(7cxP+uP)] ratios (τ_b=0.31, p<0.05). In contrast, no significant correlation was observed between the CARS scores and 7cxP/uP ratios (τ_b=0.16, p=0.26).

Table 3 summarizes an assessment of transsulfuration metabolites among the participants with ASD in comparison to the controls examined in the present study. Overall, it was observed that the participants with an ASD had significantly decreased plasma levels of cysteine, reduced glutathione, and sulfate. The abnormalities were greatest for sulfate (52% reduction in comparison with controls), with less difference in reduced glutathione and cysteine (26% and 23% reduction in comparison with controls, respectively). In contrast,

participants with ASDs had significantly increased plasma oxidized glutathione (31% increased in comparison with controls).

Table 4 summarizes an assessment of plasma oxidized glutathione levels among participants with low urinary porphyrins in comparison to participants with high urinary porphyrins. It was observed that there were significantly increased median levels of plasma oxidized glutathione among those study participants with high levels of 5cxP/g creatinine or prcP/g creatinine in comparison to participants with low levels of each of these metabolites. In contrast, the plasma oxidized glutathione levels did not significantly vary in relation to the other urinary porphyrins examined.

4. Discussion

The overall results of the present study showed decreased transsulfuration metabolites/increased urinary porphyrin metabolites associated with mercury susceptibility/toxicity in a cohort of participants diagnosed with an ASD. Furthermore, a significant correlation was found between the clinical severity of participants diagnosed with an ASD, as measured/indicated by the CARS, and urinary porphyrins associated with mercury toxicity. Finally, a significant relationship was observed between increasing levels of plasma oxidized glutathione and increasing urinary porphyrins associated with mercury toxicity.

The significant decrease in plasma reduced glutathione and increased oxidized glutathione among the participants diagnosed with an ASD relative to neurotypical controls, as well as the significantly increased plasma oxidized glutathione levels among participants with high levels of mercury-associated urinary porphyrins in comparison to participants with low levels of mercury-associated urinary porphyrins, are both of concern. Glutathione is a tripeptide of cysteine, glycine, and glutamate that is synthesized in every cell of the body. The essential intracellular reducing environment is maintained by the high ratio of reduced glutathione to the oxidized form of glutathione [27]. The reduced/oxidized glutathione redox equilibrium regulates a pleiotropic range of functions that include nitrogen and oxygen free radical scavenger [28], protein redox status and enzyme activity [29], cell membrane integrity and signal transduction [30,31], transcription factor binding and gene expression [32], phase II detoxification [33], and apoptosis [34].

Under normal physiologic conditions, glutathione reductase enzyme activity is sufficient to maintain the high reduced/oxidized glutathione redox ratio. However, excessive intracellular oxidative stress that exceeds the capacity of glutathione reductase will result in oxidized glutathione export to the plasma in attempt to regain intracellular redox homeostasis. Thus, an increase in plasma oxidized glutathione is a strong indication of intracellular oxidative stress. Further, oxidized glutathione export represents a net loss of glutathione to the cell and increases the requirement for cysteine, the rate-limiting amino acid for glutathione synthesis. Of possible relevance, plasma cysteine levels were significantly reduced in almost 40% of the participants diagnosed with ASDs. 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 preformed cysteine [35].

The significant decrease in plasma cysteine and plasma glutathione and the increase in plasma oxidized glutathione observed in the study participants with ASDs suggest that precursor availability is insufficient to maintain glutathione levels and normal redox homeostasis. Furthermore, it is apparent that increased mercury body-burden and toxicity may significantly contribute to the overall abnormal transsulfuration pathology observed among participants diagnosed with an ASD, as increased levels of plasma oxidized glutathione were found to be correlated with increased level of mercury-associated urinary porphyrins. Consistent with low glutathione levels and increased oxidative stress, children having an ASD would be expected to have

Table 4
An assessment of plasma oxidized glutathione levels among the study participants with low urinary porphyrins[a] in comparison to participants with high urinary porphyrins[b]

Lab test	Low porphyrin cases (n=14) plasma oxidized glutathione mean±Std [median]	High porphyrin cases (n=14) plasma oxidized glutathione mean±Std [median]	p-value[c]
Uroporphyrins I and III[d]	0.42±0.17[e] [0.41]	0.49±0.14 [0.52]	NS
Heptacarboxyporphyrin	0.47±0.19 [0.46]	0.44±0.12 [0.44]	NS
Hexacarboxyporphyrin	0.46±0.16 [0.46]	0.45±0.15 [0.45]	NS
Pentacarboxyporphyrin	0.39±0.14 [0.41]	0.52±0.14 [0.54]	<0.05
Precoproporphyrin	0.39±0.15 [0.37]	0.52±0.13 [0.52]	<0.05
Coproporphyrins I and III	0.42±0.15 [0.45]	0.49±0.16 [0.52]	NS

Std = standard deviation.
NS = not significant.
  [a] Low urinary porphyrin is defined as any study participant with a urinary porphyrin less than the overall study participant median.
  [b] High urinary porphyrin is defined as any study participant with a urinary porphyrin greater than the overall study participant median.
  [c] The unpaired non-parametric Mann Whitney U test statistic was utilized (two-tailed).
  [d] nmol/g creatinine.
  [e] Plasma oxidized glutathione (nmol/L).

difficulty resisting infection, resolving inflammation, and detoxifying environmental contaminants. Indeed, patients diagnosed with an ASD were reported to suffer from recurrent infections [36], neuroinflammation [37], gastrointestinal inflammation [38,39], and impaired antioxidant and detoxification capacity [40–42].

Furthermore, one should note that an important relationship between glutathione availability and mercury excretion has been found [15]. Bile is the body's main route of elimination for many metals. For example, the rate of secretion of methyl and inorganic mercury into bile was low in suckling rats but rapidly increased to adult rates soon after weaning. These changes closely paralleled similar developmental changes in the biliary secretion of reduced glutathione. Furthermore, when reduced glutathione secretion into bile was completely inhibited, without changing hepatic levels of reduced glutathione or mercury, mercury secretion was also completely blocked. These investigators concluded that their results indicated a close correspondence between the secretion of mercury and the availability of reduced glutathione in the bile.

In addition, the finding of significantly decreased plasma sulfate among participants diagnosed with ASDs in comparison to neurotypical controls is concerning. Alberti et al. showed impaired sulfation capacity in patients diagnosed with an ASD [43]. Others have shown similarly reduced sulfation products among patients diagnosed with an ASD [44]. Decreased sulfation capacity can result in decreased detoxification [45]. Within the population diagnosed with an ASD, the apparent inability to properly respond to toxins may be due, in part, to an undersupply of sulfate substrate for the sulfotransferases, resulting in impaired sulfur-dependent detoxification pathways [46]. Sulfate is essential for detoxification and plays a critical role in heavy-metal detoxification [16].

The brain has sulfate transporters, which are expressed most highly in the cerebellum and hippocampus, suggesting that, in these locations, important processes needing sulfate regulation are taking place [47]. In addition, 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 [48], probably because the supply of sulfate is so vital to the function in that region. The hippocampus and the cerebellum are the two places that have received attention from brain studies in patients diagnosed with an ASD because there is evidence of structural abnormalities in these areas [49].

Research in rats has also shown gender differences in detoxification, with females excreting significantly higher levels of mercury than males [50,51]. Other studies found that males are more dependent on sulfotransferase activity for the removal of xenobiotics [52]. In addition, investigators reported cystathionine β-synthase (CBS), which catalyzes the committing step in the transsulfuration pathway, is down-regulated by testosterone in human cells. The result is a significant decrease in flux through the transsulfuration pathway and lower intracellular glutathione levels [53]. Furthermore, in some animal models and in human fetal/infant populations, low-dose mercury exposures induced significant increases in neurotoxic effects in males relative to females having comparable mercury exposures [54]. Overall, these observations may be particularly important to patients diagnosed with an ASD because: a) the male/female ratio in those with an ASD diagnosis is at least 3:1 [1] and b) investigators have reported significant increases in testosterone in patients diagnosed with an ASD [55].

Because sulfate and glutathione are essential for effective detoxification, the effects of a lack of availability of 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 to result in developmental neurotoxicity [56].

Reduced availability of these key biochemical metabolites 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 metals. Evidence shows that metal ions disrupt glutathione production [57]. 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 rapidly depleted as a result of these heavy-metal-induced demand increases.

The overall findings made in the present study regarding the relative levels of transsulfuration metabolites measured in participants diagnosed with an ASD in comparison to controls are in agreement with the differences observed in previous studies [58–62]. Like the current study, these previous studies have shown that, relative to the controls, individuals with an ASD had significant reductions in blood levels of glutathione, cysteine, and sulfate, whereas the level of plasma oxidized glutathione was significantly increased. It was observed, when comparing the actual numeric values from the current study with previous studies, that there were some differences in the actual values measured for the different metabolites examined. This may reflect differences in the exact methods employed in measuring various blood levels of transsulfuration metabolites, but given the consistency observed between the studies, helps to indicate the overall validity of the observations.

Previous studies have shown porphyrins are heme precursors formed in the heme synthesis pathway and have found that certain abnormal porphyrins profiles afford a measure of mercury exposure [17,19,63,64]. Based on the outcomes observed, the steps in the heme pathway most vulnerable to heavy-metal inhibition are those that involve the enzymes uroporphyrin decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX). Mercury toxicity has been demonstrated to be associated with elevations in urinary cP, 5cxP, and by the expression of an atypical porphyrin prcP (also known as keto-isocoproporphyrin) not found in urine in unexposed controls [17]. Woods noted that these distinct changes in urinary porphyrin concentrations were observed as early as 1–2 weeks after initiation of mercury exposure, and that these changes increased in a dose- and time-related fashion with the concentration of mercury in the kidney, one of the principal target organs of mercury compounds [17]. In addition, urinary porphyrin profiles were also shown to correlate significantly with mercury body-burden and with specific neurobehavioral deficits associated with low-level mercury exposure [17,19,63,64]. These studies found that urinary porphyrin profiles are a useful biomarker for mercury exposure and its potential adverse health effects in human subjects [17,19,63,64].

Several previous studies have examined urinary porphyrin profiles in individuals diagnosed with an ASD [19,63,64]. The results of this present study are consistent with several other studies of individuals diagnosed with an ASD. Compatible with the results of the present study is the observation that mercury-associated urinary porphyrin profiles were found to significantly increase across the autism spectrum from individuals with a mild ASD diagnosis to those with a severe ASD diagnosis. Previous studies also demonstrated that chelation therapy in those with an ASD diagnosis resulted in significant reductions in mercury-associated urinary porphyrin profiles. The results of the present study contextualize these previous findings by, for the first time, evaluating patients diagnosed with an ASD using CARS, a recognized test of ASD severity, prior to blinded lab testing, and finding that there was a significant increasing correlation between mercury-associated urinary porphyrin profiles and ASD severity. In contrast, the urinary porphyrins that are not associated with mercury toxicity did not correlate with the child's autism severity score (see Fig. 2). The present study identifies a human clinical biomarker that correlates increasing mercury-associated toxicity with increasing ASD symptom severity.

The results of the present study are also supported by observations made in other studies on individuals diagnosed with an ASD. Specifically, the urinary porphyrin results observed in the present study, showing an increased mercury toxic effect in individuals

diagnosed with an ASD, are compatible with previous data showing, among individuals diagnosed with an ASD relative to controls: increased brain mercury levels [65]; increased blood mercury levels [66]; increased mercury levels in baby teeth [67]; decreased excretion of mercury through first baby haircuts [68]; and increased mercury in the urine/fecal samples following chelation therapy [69]. Furthermore, the about 2- to 3-fold significantly increased levels of mercury associated with urinary porphyrins are quantitatively compatible with the increased levels of mercury observed in the aforementioned studies. Finally, the results of the present study showing a significant correlation between increasing plasma oxidized glutathione (a measure of oxidative stress) and increasing mercury-associated urinary porphyrins (a measure of mercury body-burden and toxicity) are consistent with a previous brain autopsy study of patients diagnosed with an ASD, showing a significant correlation between brain levels of oxidative stress and mercury [65].

5. Strengths and limitations

The present study has a 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 ensures that biasing factors regarding clinical or lab assessments of individual participants were minimized because neither group was aware of the other's results. Second, since the present study was conducted at the ATC, a non-biomedical treatment 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. Finally, the directions of the significant effects observed were all in the biologically plausible directions, which is very unlikely to be a random occurrence.

In considering the potential limitations of the present study, the number of study participants was of moderate size. Despite this potential limitation in the present study, it was observed that there were consistent statistically significant effects. It would be worthwhile to evaluate the consistency of the results observed here with those in different and expanded cohorts of individuals diagnosed with an ASD. In addition, it would be of value in future studies to examine if there were potential correlations between other biomarkers of oxidative stress or heavy-metal toxicity and transsulfuration biomarkers among individuals diagnosed with an ASD.

6. Conclusion

The present study is the first prospective study conducted to evaluate urinary porphyrin and transsulfuration metabolites in a cohort of patients diagnosed with an ASD using routinely available clinical lab testing. For the study participants examined, this study found that increasingly severe ASDs correlated with increasing urinary porphyrins-associated with mercury toxicity. In addition, these same study participants were observed to have significant decreased levels of the transsulfuration metabolites of cysteine, sulfate, and reduced glutathione. In contrast, they also had significant evidence of increased levels of the transsulfuration metabolite of oxidized glutathione. In addition, it was observed that increasing plasma oxidized glutathione levels were correlated with increasing mercury-associated urinary porphyrins. We recommend that future studies should focus on further evaluating urinary porphyrins and transsulfuration metabolites in an expanded cohort of individuals diagnosed with an ASD, and possible treatment protocols be evaluated for their potential to correct the urinary porphyrin and transsulfuration abnormalities observed in the present study. Finally, since the lab testing employed in the present study for examining urinary porphyrins and transsulfuration metabolites is clinically available, relatively inexpensive, and relatively noninvasive, we recommend that patients diagnosed with an ASD should be routinely tested for these substances to evaluate their levels.

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