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 ≥ 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.

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

<table>
<thead>
<tr>
<th>Descriptive information</th>
<th>Overall (n=28)</th>
<th>Mild ASD* (n=14)</th>
<th>Severe ASDb (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex/age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female (ratio)</td>
<td>23/5 (4.6:1)</td>
<td>12/2 (6:1)</td>
<td>11/3 (3.7:1)</td>
</tr>
<tr>
<td>Mean age in years ± Std (range)</td>
<td>5.8 ± 2.7 (2–13)</td>
<td>6.2 ± 3.1 (3–13)</td>
<td>5.4 ± 2.2 (2–9)</td>
</tr>
<tr>
<td>Race (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>71.4% (20)</td>
<td>78.6% (11)</td>
<td>64.3% (9)</td>
</tr>
<tr>
<td>Minoritiesc</td>
<td>28.6% (8)</td>
<td>21.4% (3)</td>
<td>35.7% (5)</td>
</tr>
<tr>
<td>Autistic disorder charactercistics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean CARS Score ± Std (range)</td>
<td>38.2 ± 5.7</td>
<td>33.6 ± 3.1</td>
<td>42.8 ± 3.6</td>
</tr>
<tr>
<td>Regressive (n)c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(30–35)</td>
<td>57.1% (16)</td>
<td>57.1% (8)</td>
<td>57.1% (8)</td>
</tr>
<tr>
<td>Non-regressive (n)</td>
<td>42.9% (12)</td>
<td>42.9% (6)</td>
<td>42.9% (6)</td>
</tr>
<tr>
<td>Autism (n)</td>
<td>71.4% (20)</td>
<td>64.3% (9)</td>
<td>78.6% (11)</td>
</tr>
<tr>
<td>Autism spectrum disorders (n)d</td>
<td>28.6% (8)</td>
<td>35.7% (5)</td>
<td>21.4% (3)</td>
</tr>
<tr>
<td>Previous treatments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplements (n)</td>
<td>33.3% (9)</td>
<td>21.4% (3)</td>
<td>42.9% (6)</td>
</tr>
</tbody>
</table>

Std = standard deviation.

* 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.

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

<table>
<thead>
<tr>
<th>Lab test</th>
<th>Mild ASD cases (n=14) mean±Std [median]</th>
<th>Severe ASD cases (n=14) mean±Std [median]</th>
<th>p-valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uroporphyrins I and III</td>
<td>25.52±17.77 [22]</td>
<td>23.24±6.68 [23]</td>
<td>NS</td>
</tr>
<tr>
<td>Heptacarboxyprotoporphyrin</td>
<td>4.41±1.44 [4.9]</td>
<td>4.92±1.77 [4.2]</td>
<td>NS</td>
</tr>
<tr>
<td>Hexacarboxyprotoporphyrin</td>
<td>0.91±0.6 [0.76]</td>
<td>0.91±0.52 [0.96]</td>
<td>NS</td>
</tr>
<tr>
<td>Pentacarboxyprotoporphyrin</td>
<td>4.92±2.79 [4.4]</td>
<td>6.0±1.58 [6.2]</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pporphoporphyrin</td>
<td>17.3±8.9 [15]</td>
<td>24.05±7.28 [23]</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Coproporphyrins I and III</td>
<td>237.490 [196.5]</td>
<td>285±92.1 [257]</td>
<td>NS</td>
</tr>
<tr>
<td>Ratios</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heptacarboxyprotoporphyrin/</td>
<td>0.18±0.05 [0.16]</td>
<td>0.22±0.09 [0.20]</td>
<td>NS</td>
</tr>
<tr>
<td>Uroporphyrins I and III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexacarboxyprotoporphyrin/</td>
<td>0.04±0.03 [0.03]</td>
<td>0.04±0.02 [0.04]</td>
<td>NS</td>
</tr>
<tr>
<td>Uroporphyrins I and III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentacarboxyprotoporphyrin/</td>
<td>0.19±0.06 [0.185]</td>
<td>0.27±0.08 [0.28]</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Uroporphyrins I and III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coproporphyrins I and III/</td>
<td>0.68±0.28 [0.665]</td>
<td>1.09±3.60 [1.1]</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Uroporphyrins I and III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Precoproporphyrin+</td>
<td>9.54±2.97 [9.7]</td>
<td>12.64±1.47 [12.54]</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pentacarboxyprotoporphyrin</td>
<td>(Precoproporphyrin+</td>
<td>0.73±0.26 [0.27]</td>
<td>1.1±0.29 [1.12]</td>
</tr>
<tr>
<td>Creatinine (mg/L)</td>
<td>977±427 [867.5]</td>
<td>925±491 [954]</td>
<td>NS</td>
</tr>
</tbody>
</table>

Std = standard deviation.

[NS = not significant.]

* 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).
yielded the following results with HPLC (n the procedure of Chattaraji and Das [24]. A Shimadzu Model 646 preservative solution. Sulfate in these samples was determined using immediately after venipuncture by adding collected plasma to a (2) Ellman’s test [23]. Deming regression analysis with both methods (1) HPLC method with dual electrochemical detection [22] and This method was validated by two independent methods including: spectrophotometrically without the addition of dithiothreitol [21]. Cysteine was measured low redox potential under reducing condition. Cysteine was measured in the samples provided by the participants with an ASD diagnosis were compared to prospective samples collected by testing neurological bo simple boys and girls between the ages of 2–16 years of age by the lab. Significant sex-specific differences were not observed among the 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. Transulfuration 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>
<thead>
<tr>
<th>Lab test</th>
<th>Mean±Std overall ASD participants (n=28)</th>
<th>Mean±Std controls a (n)</th>
<th>p-value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma cysteine (µmol/L)</td>
<td>17.8±1.9</td>
<td>23.2±4.2 (64)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma reduced glutathione</td>
<td>3.1±0.52</td>
<td>4.2±0.72 (120)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma oxidized glutathione (µmol/L)</td>
<td>0.46±0.16</td>
<td>0.35±0.05 (120)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Plasma total sulfate (µmol/g P)</td>
<td>924±245</td>
<td>1930±184 (82)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Std = standard deviation.

a Prospective samples collected by testing neurological 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 \( \leq 0.05 \) 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.

It was found that there were significant correlations between the CARS scores and specific urinary porphyrin levels among the participants examined. There was no significant correlation observed between the CARS scores and cP/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 between the CARS scores and specific urinary porphyrin levels, with the 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).

#### Table 2

<table>
<thead>
<tr>
<th>Lab test</th>
<th>Low porphyrin cases (n=14)</th>
<th>High porphyrin cases (n=14)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uroporphyrins I and IIla</td>
<td>0.42±0.17e [0.41]</td>
<td>0.49±0.14 [0.52]</td>
<td>NS</td>
</tr>
<tr>
<td>Heptacarboxy whole porphyrins</td>
<td>0.47±0.19 [0.46]</td>
<td>0.44±0.12 [0.44]</td>
<td>NS</td>
</tr>
<tr>
<td>Hexacarboxy whole porphyrins</td>
<td>0.46±0.16 [0.46]</td>
<td>0.45±0.15 [0.45]</td>
<td>NS</td>
</tr>
<tr>
<td>Pentacarboxy whole porphyrins</td>
<td>0.39±0.14 [0.41]</td>
<td>0.52±0.14 [0.54]</td>
<td>-0.05</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td>0.39±0.15 [0.37]</td>
<td>0.52±0.13 [0.52]</td>
<td>-0.05</td>
</tr>
<tr>
<td>Coproporphyrins I and III</td>
<td>0.42±0.15 [0.45]</td>
<td>0.49±0.16 [0.52]</td>
<td>NS</td>
</tr>
</tbody>
</table>

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).

### 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 and 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
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 blocked, 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 capacity [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 [11] 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, SCp, and by the expression of an atypical porphyrin pCp (also known as keto-isocopro)porphyrin 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 porphyrins 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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