



Original Article

Toxicity biomarkers in autism spectrum disorder: A blinded study of urinary porphyrins

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Abstract *Background:* Recent studies suggest that children diagnosed with an autism spectrum disorder (ASD) have significantly increased levels of urinary porphyrins associated with mercury (Hg) toxicity, including pentacarboxyporphyrin (5cxP), precoproporphyrin (prcP), and coproporphyrin (cP), compared to typically developing controls. However, these initial studies were criticized because the controls were not age- and gender-matched to the children diagnosed with an ASD. *Methods:* Urinary porphyrin biomarkers in a group of children (2–13 years of age) diagnosed with an ASD ($n = 20$) were compared to matched (age, gender, race, location, and year tested) group of typically developing controls ($n = 20$). *Results:* Participants diagnosed with an ASD had significantly increased levels of 5cxP, prcP, and cP in comparison to controls. No significant differences were found in non-Hg associated urinary porphyrins (uroporphyrins, hexacarboxyporphyrin, and heptacarboxyporphyrin). There was a significantly increased odds ratio for an ASD diagnosis relative to controls among study participants with precoproporphyrin (odds ratio = 15.5, $P < 0.01$) and coproporphyrin (odds ratio = 15.5, $P < 0.01$) levels in the second through fourth quartiles in comparison to the first quartile. *Conclusion:* These results suggest that the levels of Hg-toxicity-associated porphyrins are higher in children with an ASD diagnosis than controls. Although the pattern seen (increased 5cxP, prcP, and cP) is characteristic of Hg toxicity, the influence of other factors, such as genetics and other metals cannot be completely ruled out.

Key words autism, autism spectrum disorder, heavy metal, mercury, porphyrins, toxicity.

Introduction

An autism spectrum disorder (ASD) is a neurological disorder that limits a person's ability to function normally. Behavioral abnormalities, social limitations, sensory processing abnormalities, and impaired ability to communicate are the main issues in these multifaceted disorders, which range in clinical symptoms, from severe to mild among individuals diagnosed with autistic disorder (autism), pervasive developmental delay not otherwise defined (PDD-NOS), to Asperger's disorder.^{1,2}

Although the role of mercury (Hg) in the pathology of autism is still being debated, many studies suggest that Hg levels are higher in children with autism than in typically developing children (controls), e.g. studies that examine Hg levels in hair, blood, urine, and teeth.³ A more recent approach is to use urinary porphyrins as measure of Hg body-burden. Previous studies have shown that urinary porphyrins (heme precursors formed in the

heme synthesis pathway, Fig. 1) can afford a measure of xenobiotic exposure, particularly Hg.^{5–7} Specific patterns of urinary porphyrins suggest the presence of Hg. Hg toxicity has been demonstrated to be associated with elevations in urinary coproporphyrin (cP), pentacarboxyporphyrin (5cxP), and by the expression of an atypical porphyrin–precoproporphyrin (prcP) (also known as keto-isocoproporphyrin) not found in the urine of unexposed controls. Woods⁵ noted that these distinct changes in urinary porphyrin concentrations were observed as early as 1–2 weeks after initiation of Hg exposure, and that they increased in a dose- and time-related fashion with the concentration of Hg in the kidney, a principal target organ of Hg compounds. In addition, urinary porphyrin profiles were also shown to correlate significantly with Hg body-burden and with specific neurobehavioral deficits associated with low level Hg exposure. Woods⁵ concluded that urinary porphyrin profiles are a useful biomarker for Hg exposure and its potential adverse health effects in human subjects.

Recent evidence suggests that the levels of Hg-associated porphyrins are different in children having a diagnosis of an ASD as compared to those levels in controls. Studies revealed that children with an ASD diagnosis had significantly increased levels

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Received 11 January 2010; revised 3 May 2010; accepted 9 June 2010.

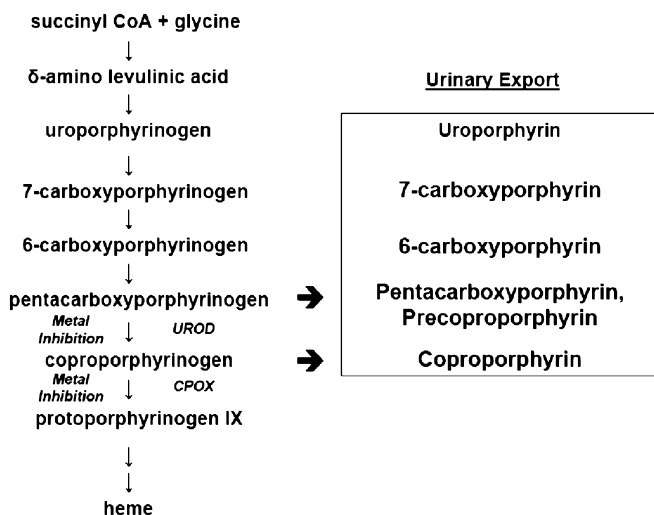


Fig. 1 A summary of the heme synthesis pathway and major urinary metabolites.⁴ Porphyrinogens appear in urine as porphyrin derivatives (right). Mercury can cause increased urinary 5cxP, prcP, and cP by inhibiting uroporphyrinogen decarboxylase (UROD) and/or coproporphyrinogen oxidase (CPOX); urinary uroporphyrin is not reported to alter with inhibition of these enzymatic steps.

of urinary 5cxP, prcP, and cP as compared to the levels in controls.^{4,8–10} In addition, chelation therapy significantly lowered the initial levels of the prcP and cP among children diagnosed with an ASD.^{4,8,9}

These initial studies were criticized because the controls were not age- and gender-matched to the children diagnosed with an ASD. The purpose of the present, blinded study was to evaluate these porphyrin biomarkers of Hg toxicity in the porphyrin pathway in a cohort of children diagnosed with an ASD as compared to age-, gender-, race-, region-of-residency-, and year-of-sample-collection-matched controls using clinically available laboratory testing.

Methods

The study was conducted at the Autism Treatment Center (Dallas, TX, USA). The study protocol received Institutional Review Board (IRB) approval from Liberty IRB Inc. (Deland, FL, USA). All parents signed a consent and Health Insurance Portability and Accountability Act (HIPAA) form and all received a copy.

The current study was designed to assess the general urinary biomarkers associated with Hg toxicity in the porphyrin pathway in a group of children having a pre-existing, independently established, clinical diagnosis of an ASD and a matched (age, gender, race, location, and year tested) group of controls.

After a confirmatory Childhood Autism Rating Scale (CARS)¹¹ evaluation on each participant in the study, a first-morning-urine sample was collected and submitted to Laboratoire Philippe Auguste (Paris, France, ISO-certified) for a standard urinary porphyrin profile assessment. The urine porphyrin results reported by this laboratory were then tabulated and examined to ascertain any differences in urinary porphyrin levels among children with an ASD diagnosis as compared to matched control children.

Participants

The present study looked at consecutive qualifying participants diagnosed with an ASD ($n = 20$) and age-, gender-, race-, region-of-residency-, and year-of-sample-collection-matched controls ($n = 20$). Age matching was defined as less than 12 months apart in age. All participants in the study lived in suburban areas of greater Dallas/Fort Worth Metroplex that is primarily comprised of Dallas, Tarrant, Collin, and Denton counties (located in north-central Texas). Children included in the present study were between 2 and 13 years of age. Children in both groups had received routine childhood vaccinations and none of the children had received chelation therapy prior to the study. Children were included in the present study with a history of taking common supplements, such as children's vitamins. None of the children included in the present study had a history of taking supplements known to be involved in detoxification, such as glutathione or glutathione precursors. None of the children included in the present study were on any unusual diets. Both groups were recruited from the community by using flyers and word of mouth.

The children with an ASD had a CARS score ≥ 30 .¹¹ A child with a CARS score ≥ 30 is considered to have autism.

The controls had CARS scores < 18 (the lowest possible CARS score is 15). The controls could not have any history of neurological, learning, medical, or developmental problems.

Furthermore, this study excluded all children with a history of Fragile X disorder, tuberous sclerosis, phenylketonuria (PKU), Lesch-Nyhan syndrome, fetal alcohol syndrome, or any history of maternal illicit drug use.

Clinical evaluation

As a baseline, the researchers involved in the present study obtained information 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 on all of the children by the Principal Investigator (PI), who was trained in the use of CARS and has 12 years' experience in using CARS to evaluate more than 300 persons with an ASD diagnosis. The PI completed the CARS evaluation by interviewing the parents and observing the child. Table 1 summarizes the pertinent demographics of the participants included in the present study.

CARS testing

The CARS is a recognized 15-item behavioral rating scale developed to identify autism as well as to quantitatively describe the severity of the disorder. A total score of 15–29.5 is considered non-autistic; a score of 30–36.5 is considered mild to moderate autism; a score from 37–60 is considered moderate to severe autism. The CARS is a well-established measure. The internal consistency reliability alpha coefficient is 0.94; the inter-rater reliability correlation coefficient is 0.71; and the test-retest correlation coefficient is 0.88. The CARS scores have high criterion-related validity when compared to clinical ratings during the same diagnostic sessions, with a significant correlation of 0.84.¹¹

Table 1 An overall summary of the subjects examined

Descriptive information	Cases [†] (<i>n</i> = 20)	Controls [‡] (<i>n</i> = 20)
Sex/age		
Male/female (ratio)	15/5 (3:1)	15/5 (3:1)
Mean age in years ± SD (range)	6.6 ± 2.78 (2–13)	6.75 ± 2.83 (3–13)
Race (<i>n</i>)		
Caucasian	60% (12)	60% (12)
Black	15% (3)	15% (3)
Hispanic	10% (2)	10% (2)
Asian	5% (1)	5% (1)
Mixed	10% (2)	10% (2)
Observed CARS characteristics		
Mean CARS score ± SD (range)	38.63 ± 5.98 (30–50)	15.32 ± 0.9 (15–18)
Regressive (<i>n</i>) [§]	65% (13)	–
Non-regressive (<i>n</i>)	35% (7)	–
Autism (<i>n</i>)	75% (15)	–
Other autism spectrum disorders (<i>n</i>) [¶]	25% (5)	–

All subjects examined in the present study were living in the greater Dallas/Fort Worth Metroplex (located in north-central Texas) and had not previously received chelation therapy.

[†]Subjects diagnosed with an ASD (CARS score ≥ 30).

[‡]Subjects that were neurotypical (CARS score ≤ 18). Matched to cases for age, gender, race, geographical location, and year of sample collection.

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

[¶]Other autism spectrum disorders include participants diagnosed with pervasive developmental disorder – not otherwise specified (PDD-NOS) or with Asperger's disorder.

SD, standard deviation.

Laboratory evaluation

Following the intake evaluation, parents were given a urinary porphyrin collection kit and instructions. The laboratory specimens were all collected as first morning urine samples following an overnight fast. Specimens were shipped to Laboratoire Philippe Auguste. The laboratory used in the present study was blinded and received no information regarding the clinical status of the participants examined or their CARS score. Urine specimens underwent routine urinary porphyrin profile testing at Laboratoire Philippe Auguste. The profile includes the following urinary porphyrins: uroporphyrin (uP), heptacarboxyporphyrin (7cxP), hexacarboxyporphyrin (6cxP), 5cxP, precP, and cP.

Laboratory methods

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 a vacuette containing a preservative (Greiner Bio-One, Les Ulis, France), maintained in the dark at ambient temperature during shipping to France (approximately 5–10 days), and then frozen (–20°C) until analysis (preserved samples collected in the Greiner Bio-One tubes used for porphyrin testing and held at room temperature have been shown to provide accurate/reproducible porphyrin result values for more than 20 days, as reported by Laboratoire Philippe Auguste). Porphyrins were determined by the HPLC spectrofluorimetric technique: after centrifugation (3000 × *g*, 5 min), 500 µL urine was acidified with 50 µL of aqueous hydrochloric acid (37% w/v), recentrifuged, and 20 µL of the resulting sample injected into Econosphere column (18, 5 µm particle size, 250 × 46 mm, Alltech, Templemars, France). Elution from the

column was effected using a gradient (Phase A: 50 mmol KH₂PO₄, pH adjusted to 3.5 with CH₃COOH; Phase B: CH₃OH). Eluant flow was 1 mL/min (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) was used for the porphyrins in the system that had dual on-line 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, precP, and cP, respectively. The HPLC separation method used by this laboratory does not separate the I and III isomers of uP and cP. The fluorescence responses from the samples were quantified against the corresponding responses from a mixed porphyrin reference sample (Porphyrin Products, Logan, UT, USA). The urinary creatinine level in each sample was measured using a spectrophotometric assay (Vitros, Ortho-Clinical Diagnostics, Johnson & Johnson). The porphyrin values reported were expressed in nanomoles per g of creatinine measured.⁴

Statistical analyses

In order to evaluate the significance of the differences in the urinary porphyrin levels measured among participants diagnosed with an ASD in comparison to the corresponding levels in the matched controls, the Wilcoxon matched-pairs signed-ranks test statistic was utilized. The null hypothesis was that there would be no difference in the data distributions for urinary porphyrin levels measured among participants diagnosed with an ASD in comparison to those for the matched controls. In addition, for those porphyrins where the differences in the results between cases and controls were observed to be statistically significant, a follow-up

statistical evaluation was undertaken utilizing the non-parametric sign test statistic. In addition, data for each specific urinary porphyrin level examined in the present study were divided into quartiles, and the odds ratio for the frequency of an ASD diagnosis in comparison to controls in the combined second, third, and fourth quartiles ($n = 30$) as compared to the first quartile ($n = 10$) utilizing the Fisher's exact test statistic were computed. The null hypothesis was that the Odds ratio for the frequency of an ASD diagnosis in comparison to controls would remain similar for each specific urinary-porphyrin quartile examined. For all statistical tests employed in the present study, a two-tailed P -value ≤ 0.05 was considered to be statistically significant.

Results

Participants diagnosed with an ASD had significantly increased urinary porphyrins result values for those porphyrins associated with Hg intoxication (the prcP and the cP components) in comparison to the corresponding values for the controls. Overall, mean prcP levels were 1.2-fold ($P < 0.05$) and mean cP levels were 1.3-fold ($P < 0.005$) increased among participants diagnosed with an ASD in comparison to controls. There were no significant differences found between the two groups of children for the results reported for the non-Hg associated porphyrins or urinary creatinine levels. A summary of these findings is reported in Table 2.

Furthermore, it was observed when excluding a single participant among the controls with urinary porphyrin values that were significant outliers from other controls (more than 2 standard deviations above the mean) and the corresponding matched participant diagnosed with an ASD, there were significant increases in mean 5cxP (1.3-fold, $P < 0.05$, ASD = 5.14 ± 1.34 nmol/g creatinine, control = 4.11 ± 1.4 nmol/g creatinine), prcP (1.3-fold, $P < 0.01$, ASD = 21.2 ± 6.96 nmol/g creatinine, control = 16.5 ± 6.86 nmol/g creatinine), and cP (1.4-fold, $P < 0.001$, ASD = 255 ± 76 nmol/g creatinine, control = 184 ± 76 nmol/g creatinine) levels among participants diagnosed with an ASD in comparison to the corresponding values for the controls. Other urinary porphyrins not associated with Hg and urinary creatinine levels remained similar between the two groups of children.

Table 3 shows the Odds ratio for an ASD diagnosis relative to controls for those study participants having result values in the second through fourth quartiles in comparison to the first quartile for each of the specific urinary porphyrin components reported by the laboratory. There were significantly increased Odds ratios for an ASD diagnosis relative to controls among study participants with prcP (Odds ratio = 15.5, $P < 0.01$) and cP (Odds ratio = 15.5, $P < 0.01$) levels in the second through fourth quartiles in comparison to the first quartile.

Discussion

The results of the present study indicate increased levels of those urinary porphyrin species associated with Hg toxicity in a cohort of participants diagnosed with an ASD relative to matched controls. As mentioned, several previous studies have examined urinary porphyrin profiles in individuals diagnosed with an ASD in comparison to controls.^{4,8-10} Geier and Geier⁸ in 2006 found elevated cP levels in children with ASD ($n = 37$) as compared to their siblings ($n = 7$). In 2007, Geier and Geier⁹ found that patients diagnosed with an ASD ($n = 71$) had significant elevations in urinary levels of cP, 5cxP, and prcP relative to controls ($n = 14$), and >50% of patients diagnosed with an ASD had urinary cP levels more than 2 standard deviations above the mean values for controls. Significant reductions in urinary 5cxP and cP levels were observed in ASD patients following chelation, and a significant relationship between the severity of the child's ASD diagnosis and the elevation of Hg-associated urinary porphyrins (i.e. the higher the Hg-associated porphyrins, the more severe the diagnosis). These two studies were conducted in the US. Similarly, Nataf *et al.*⁴ examined French children diagnosed with autism ($n = 106$) and found that these children had elevated prcP and cP as compared to controls ($n = 12$). In addition, >50% of patients diagnosed with autism had urinary prcP levels more than 2 standard deviations above the mean values for controls. They also found a significant relationship between the severity of the child's ASD diagnosis and Hg-associated urinary porphyrins. Austin and Shandley¹⁰ found similar findings in children with ASD ($n = 41$) in Australia using control data from lab reference ranges and other studies. These previously mentioned studies

Table 2 Urinary porphyrin levels among the subjects examined

Lab test	Cases-ASD [†] ($n = 20$) Mean \pm SD [range]	Controls-neurotypical [‡] ($n = 20$) Mean \pm SD [range]	P -value [§]
nmol/gram creatinine			
Uroporphyrins	24.5 \pm 7.9 [12–38]	20.7 \pm 7.7 [9.2–39]	NS
Heptacarboxyporphyrins	4.4 \pm 1.3 [2.1–7.1]	4.1 \pm 1.6 [1.7–8.7]	NS
Hexacarboxyporphyrins	1.0 \pm 0.46 [0.28–1.7]	0.89 \pm 0.42 [0.4–1.7]	NS
Pentacarboxyporphyrins	5.1 \pm 1.3 [2.7–7.2]	4.45 \pm 2.09 [2.01–11.2]	NS
Precoproporphyrins	21.1 \pm 6.8 [13.5–35]	17.9 \pm 9.3 [5.5–45.2]	<0.05 [¶]
Total coproporphyrins (I + III)	253 \pm 74.4 [163–405]	195 \pm 88 [74–399]	<0.005 [¶]
Grams of creatinine/liter	992 \pm 494 [236–1780]	1035 \pm 488 [308–2425]	NS

[†]Subjects diagnosed with an ASD (CARS score ≥ 30).

[‡]Subjects that were neurotypical (CARS score ≤ 18). Matched to cases for age, gender, race, geographical location, and year of sample collection.

[§]The two-tailed, non-parametric Wilcoxon matched-pairs signed-ranks test statistic was utilized.

[¶]The two-tailed, paired non-parametric sign test statistic reported a $P < 0.05$ difference when the cases were compared to the matched controls. NS, not significant; SD, standard deviation.

Table 3 The odds ratio of an ASD diagnosis in comparison to urinary porphyrins

Urinary porphyrin	Data quartiles	Range (nmol/g creatinine)	Odds ratio of an ASD diagnosis	P-value [†] (95% CI)
Uroporphyrin	1	9.2–16.03	1.0	Reference
	2–4	16.9–39	3.05	NS (0.54–21.3)
Heptacarboxyporphyrin	1	1.7–3.3	1.0	Reference
	2–4	3.4–8.7	1.0	NS (0.19–5.4)
Hexacarboxyporphyrin	1	0–0.6	1.0	Reference
	2–4	0.6–2.2	1.7	NS (0.32–9.9)
Pentacarboxyporphyrin	1	2.01–3.5	1.0	Reference
	2–4	3.6–11.2	3.05	NS (0.54–21.3)
Precoproporphyrin	1	5–13.4	1.0	Reference
	2–4	14–45.2	15.5	<0.01 (1.7–716)
Coproporphyrin	1	74–165	1.0	Reference
	2–4	166–405	15.5	<0.01 (1.7–716)
Creatinine (grams/L)	1	236–613	1.0	Reference
	2–4	631–2425	1.7	NS (0.32–9.9)

Urinary porphyrins levels were divided into quartiles, and the frequency of an ASD diagnosis in combined second, third, and fourth quartiles ($n = 30$) was compared to the first quartile ($n = 10$).

[†]Calculated using the Fisher's exact test statistic.

CI, confidence interval; NS, not significant.

used Laboratoire Philippe Auguste and/or Laboratory Corporation of America (LabCorp) (CLIA-approved laboratory based in the US). More recently, a urinary porphyrin study conducted in Korea, using Metamatrix (CLIA-approved laboratory based in the US), found significant increases of uP, 5cxP, prcP, cP, and total porphyrins in children diagnosed with an ASD ($n = 65$) as compared to controls ($n = 9$).¹²

Our present study was different from previous studies in that the two groups were age-, gender-, race-, region-of-residency-, and year-of-sample-collection-matched children (ASD and neurotypical). Nonetheless, the results found are consistent with those reported in the previous studies.

All of the studies show that Hg-associated porphyrins are higher in children with autism than in controls, suggesting that children with autism have a higher level of Hg toxicity. As mentioned in the introduction of this paper, the pattern seen (increased 5cxP, prcP, and cP) is characteristic of Hg toxicity because Hg toxicity has been demonstrated to be associated with elevations in urinary cP, 5cxP, and prcP (an atypical porphyrin not found in the urine in unexposed controls) (see heme synthesis pathway, Fig. 1).^{5,13} Woods⁵ noted that these distinct changes in urinary porphyrin concentrations were observed as early as 1–2 weeks after initiation of Hg exposure, and that they increased in a dose- and time-related fashion with the concentration of Hg in the kidney, a principal target organ of Hg compounds.

Elevated Hg in the kidney usually also suggests elevated Hg in the brain, as studies show that the brain and kidneys are target organs for Hg following Hg exposure. For example, Pingree *et al.*⁷ gave rats methyl-Hg hydroxide (MMH) (10 ppm) in drinking water for 9 weeks and determined both inorganic (Hg^{2+}) and organic (CH_3Hg^+) Hg species levels in urine and tissues by cold vapor atomic fluorescence spectroscopy (CVAFS). After the treatment, Hg^{2+} and CH_3Hg^+ concentrations were 0.28 and 4.80 $\mu\text{g/g}$ in the brain and 51.5 and 42.2 $\mu\text{g/g}$ in the kidney, respectively.

Hg exposure in all forms of Hg (elemental, inorganic, and organic) is known to cause neural degeneration. However, what

may be less known is that mechanisms for neuronal degeneration are comprehensive and wide-ranging. There does not appear to be single target effect, but a plethora of consequences and cascades of events. The following lists some of the neurological consequences of Hg exposure in the brain: (i) inhibition of the binding of GTP to tubulin, thereby inhibiting tubulin polymerization into microtubules;¹⁴ (ii) growth cone collapse;¹⁴ (iii) disruption of the membrane structure and linear growth rates;¹⁴ (iv) disintegrated tubulin/microtubule structure;¹⁴ (v) neuronal somata sprouting failure;¹⁴ (vi) increase of lipid peroxidation (LPO) or oxidative stress;^{15,16} (vii) excitotoxicity, increased cytosolic free calcium, and cell death;¹⁷ (viii) disruption in insulin-like growth factor 1 (IGF-1) signaling and methionine synthase activity in the brain;¹⁸ (ix) disruption of the postnatal development of the glutathione antioxidant system in the brain;¹⁹ (x) reduced glutathione level in the brain and a decrease in the enzymatic activities of acetyl cholinesterase in different regions of the brain;¹⁶ and (xi) immune-glutamatergic dysfunction.^{20,21}

Although the role of Hg exposure in autism is still being debated with some conflicting research findings, many studies that examine toxic metal levels in children diagnosed with an ASD in comparison to controls find that children diagnosed with an ASD have increased levels of heavy metals. For example, DeSoto and Hitlan²² analyzed a cohort of children from China (ASD and controls) for their blood Hg levels and found that the children diagnosed with an ASD had higher blood levels of Hg than controls. Adams *et al.*²³ found that children diagnosed with an ASD had higher levels of Hg in their baby teeth. Many other studies have shown differences in toxic metal levels in the children diagnosed with autism compared to controls.^{24–33}

Furthermore, it was observed in a blinded study of children diagnosed with an ASD that the greater the Hg body burden (as measured by urinary porphyrins) the more severely affected the child's autism symptoms, as measured by a professional evaluation using the CARS.³⁴ It was also observed from regression analyses that the body burden of toxic metals, particularly Hg, as assessed by urinary excretion before and after detoxification

therapy, was significantly related to autism severity, as measured using the Autism Diagnostic Observation Schedule, among children diagnosed with an ASD.³⁵

By contrast, a few other studies have failed to find a significant association between Hg and an ASD diagnosis. For example, investigators observed, in a non-biologically plausible finding, that without adjustment, children diagnosed with an ASD had a significant reduction in their blood Hg levels in comparison to neurotypical controls (32% reduction in blood Hg levels), and this same effect was apparent for children diagnosed with developmental delay (39% reduction in blood Hg levels).³⁶ Another study reported on the use of the oral chelator meso-2,3-dimercaptosuccinic acid (DMSA) to be used diagnostically to mobilize heavy metals from extravascular pools, enhancing the identification of individuals who have a chelatable body burden.³⁷ Fifteen children with autism and four controls successfully completed a pilot study to test for chelatable body burden of arsenic (As), cadmium (Cd), lead (Pb), and Hg. These investigators observed that three subjects diagnosed with autism excreted one metal in greater quantity during the provoked excretion than baseline (20% of the children diagnosed with autism examined), and one of these children (6.7% of the children diagnosed with autism examined) was observed to have provoked excretion of Hg between the upper limit of normal and lower limit of the potentially toxic reference range. None of controls had excreted any metal in greater quantity during the provoked excretion than baseline. Despite these observations, the investigators concluded that the proportion of autistic participants in this study whose DMSA provoked excretion results demonstrate an excess chelatable body burden of As, Cd, Pb, or Hg is zero. Other investigators examined blood Hg levels in children diagnosed with an ASD in comparison to controls, and found no difference in the mean Hg levels. These investigators concluded that there is no causal relationship between Hg and autism.³⁸ Subsequently, these investigators acknowledged that their article contained significant statistical errors and contained, as explained by the investigators, “unintentional careless mistakes”.³⁹ Considering these previous studies observed in some cases non-biological plausible results, may have potentially suspect conclusions, and have acknowledged significant methodological errors, it is hard to interpret these previous investigators’ findings. The evidence from the present study, along with emerging evidence from multiple lines of clinical observation of patients diagnosed with an ASD, supports an association between Hg and an ASD diagnosis.

Study limitations

As mentioned earlier, the pattern seen (increased 5cxP, prcP, and cP) is characteristic of Hg toxicity. However, the influence of other factors, such as other heavy metals (e.g. Pb) cannot be completely ruled out. For example, lead is known to increase cP, therefore the possibility of the presence of lead contributing to the higher levels of cP would need to be considered.⁴⁰ In addition, there are several possible sources of Hg exposure and specific sources of Hg or other metal exposure could not be determined from this study. The possible role of genetics would also need to be considered. Evidence from previous research suggests suscep-

tibility in children with autism due to problems in the transsulfuration pathway and glutathione synthesis (glutathione is critical for heavy metal detoxification).⁴¹ Buyske *et al.*⁴² for example, found a genotype relative risk near 2 for the homozygous glutathione S-transferase M1 (GSTM1) deletion genotype and autism.

Study strengths

The study was a prospective, cross-sectional study of consecutive children. The children were age-, gender-, race-, region-of-residency-, and year-of-sample-collection-matched. Two different analyses were conducted and both yielded statistically significant results. All specimens were collected, shipped, and analyzed the same way. The laboratory was blinded to group assignment.

Conclusion

The results of this present study suggest that children diagnosed with an ASD have higher levels of Hg-related porphyrins, which implies that they have higher levels of Hg body-burden. Taken together with the previously mentioned studies, this study suggests that children with an ASD are poor detoxifiers relative to typically developing children, and therefore, may be vulnerable to toxic metal accumulation and the physiological consequences of it.

Acknowledgments

This research was funded by a grant from the Autism Research Institute, non-profit CoMeD, Inc., and by the non-profit Institute of Chronic Illnesses, Inc. through a grant from the Brenen Hornstein Autism Research & Education (BHARE) Foundation. The authors wish to acknowledge the help of the parents and children who participated in the study; without their participation this type of investigation would not be possible.

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