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A Prospective Assessment of Porphyrins in Autistic Disorders: A Potential Marker for Heavy Metal Exposure

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Autism was recently associated with a urinary porphyrin pattern indicative of mercury toxicity in a large cohort of French children. The IRB of the Institute for Chronic Illnesses approved the present study. A total of 37 consecutive American patients (≥7 years-old) with autism spectrum disorders (ASDs) (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition-DSM IV), born from 1983-1998, that presented to the Genetic Centers of America for outpatient genetic evaluations were prospectively examined for urinary prophryin levels (LabCorp, Inc.) from June 2005-June 2006. Imaging and laboratory testing were conducted on each patient to rule-out other causal factors for their ASDs. As controls, age-, sex-, and race-matched neurotypical ASD siblings were examined. An apparent dose-response effect was observed between autism severity and increased urinary coproporphyrins. Patients with non-chelated autism (2.25-fold, 83% had levels > 2 SD above the control mean) and non-chelated ASDs (2-fold, 58% had levels > 2 SD above the control mean), but not patients with non-chelated pervasive developmental delay-not otherwise specified (PDD-NOS) or Asperger's disorder (1.4-fold, 46% had levels > 2 SD above the control mean), had significantly increased median coproporphyrin levels versus controls. A significant increase (1.7-fold) in median coproporphyrin levels was observed among non-chelated ASD patients versus chelated ASD patients. Porphyrins should be routinely clinically measured in ASDs, and potential ASD treatments should consider monitoring porphyrin levels. Additional research should be conducted to evaluate the potential role for mercury exposure in some ASDs.

Keywords: Autistic; Chelation; Developmental Delay

INTRODUCTION

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impairments in social relatedness and communication, repetitive behaviors, abnormal movement patterns, and sensory dysfunction (Eigsti and Shapiro, 2003; Werner and Dawson, 2005). While genetic factors are recognized as being important in the pathogenesis of ASDs, a role for environmental factors has received considerable attention. Several recent epidemiological studies have associated mercury exposure with ASDs (Counter *et al.*, 2002; Holmes *et al.*, 2003; Geier and Geier, 2005; Palmer *et al.*, 2006; Windham *et al.*, in press), and it has been reported that exposure to mercury can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining or associated with ASDs, and that these similarities extend to neuroanatomy, neurotransmitters, and biochemistry (Faustman *et al.*, 2000; Bernard *et al.*, 2001; 2002; Redwood et al, 2001; Blaxill *et al.*, 2004). Furthermore, a recent review has suggested that ASD children have been found to have significantly higher exposure to mercury than controls, and ASD children have been determined to have significantly increased body-burdens of mercury resulting from biochemical and genomic susceptibilities within detoxification pathways (Mutter *et al.*, 2005).

Recently, Nataf *et al.* (2006) have examined urinary porphyrin levels in a large series of children with autistic disorders from France. These researchers observed a porphyrin pattern among children with ASDs that implicated environmental toxicity, especially mercury toxicity, in childhood autistic disorders. The purpose of the present study was to conduct an examination of urinary porphyrin levels in a series of American patients with ASDs, so as to evaluate the consistency of the observations made by Nataf *et al.* in France with clinical observations made in the United States.

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MATERIALS AND METHODS

The Institutional Review Board of the Institute for Chronic Illnesses (Office for Human Research Protections, US Department of Health and Human Services IRB number: IRB00005375) approved the present study.

Subjects

Study subjects were consecutive patients (≥ 7 years-old) with ASDs that prospectively presented to the Genetic Centers of America for outpatient genetic evaluations from June 2005-June 2006. Each patient was previously diagnosed with an ASD based upon the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. A total of 37 patients with ASDs were identified that were born from 1983 through 1998. Table I summarizes the overall profile of the patients with ASDs examined in the present study. Each patient's medical records were reviewed to assess chelation status. Patients were considered non-chelated if they had never been chelated, whereas chelated patients were those who had previously documented in their medical records chelation therapy that lasted longer than 3 months. Each patient was also tested to rule-out brain structural abnormalities (CT or MRI head scans). In addition, laboratory testing was conducted on each patient, and all were determined to be negative for Fragile X Syndrome, chromosomal abnormalities (structural and numeric), subtelomere chromosome rearrangements, thyroid function abnormalities, Pradder Willi Syndrome/Angelman, urine organic acid abnormalities, Polychlorinated Biphenyl/pesticide exposure, and Rett Syndrome (LabCorp, Inc.).

Evaluation

Each patient was tested for urine porphyrin markers (LabCorp, Inc.), including (these samples were collected as first morning urine samples): uroporphyrin,

Table I Study group profile of patients examined in the present study that presented for outpatient care to the Genetic Centers of America from June 2005 through June 2006

	Autistic Group	Control Group
Number of males/females (male/female ratio)	33/4 (8.25:1)	6/1 (6:1)
Median age in years (range)	11 (7-22)[a]	12 (7-20)
Median year of birth (range)	1995 (1983-1998)[a]	1993 (1985-1998)
Autism (n)	38% (14)	NA
Autistic Disorders (n)[b]	62% (23)	NA
Racial Demographic		
Caucasians (n)	84% (31)	86% (6)
Minorities (n)[c]	16% (6)	14% (1)
Residence[d]		
East Coast (n)	59% (22)	71% (5)
Central (n)	30% (11)	33% (2)
West Coast (n)	11% (4)	0% (0)
Treatment Status		
Previously Chelated[e]	49% (18)	0% (0)
Previously Non-chelated	51% (19)	100% (7)

NA = Not Applicable

[a] Differences observed were not statistically significantly different based upon the non-parametric Mann-Whitney *U* test statistic.

[b] Includes patients only diagnosed with pervasive developmental delay-not otherwise specified (PDD-NOS) or Asperger's disorder.

[c] Minorities include: Blacks and Indians

[d] East Coast: MD, NC, PA, SC, VA. Central: IA, IL, KS, KY, TN. West Coast: CA, WA.

[e] Patients past medical history indicated that they had previously received chelation treatment for > 3 months.

heptacarboxylporphyrin, hexacarboxylporphyrin, pentacarboxylporphyrin, and coproporphyrin.

Controls

Age-, sex- and race-matched neurotypical siblings of ASD patients were used as controls to evaluate the results obtained from the ASD patients in the present study. Table I summarizes the overall profile of the controls examined in the present study.

Statistical Analyses

In the present study, the statistical package contained in StatsDirect (Version 2.4.2) was employed. The non-parametric Mann-Whitney *U* test statistic was utilized to determine statistical significance. A two-tailed *p* value ≤ 0.05 was considered statistically significant.

RESULTS

Table II summarizes the urine porphyrin markers observed among the different patient populations examined in the present study. Among the urinary porphyrin markers examined, it was found that there were non-significant differences in the median levels of uroporphyrin, heptacarboxylporphyrin, hexacarboxylporphyrin, and pentacarboxylporphyrin among the patients examined in the present study.

It was found that those with non-chelated autism had significantly increased (2.25-fold) median coproporphyrin levels in comparison to controls, with 83% of

Table II A summary of the urine porphyrin markers observed among the patients examined in the present study.

	Uroporphyrin (μg/l)[a]	Heptacarboxylporphyrin (μg/l)	Hexacarboxylporphyrin (μg/l)	Pentacarboxylporphyrin (μg/l)	Coproporp (μg/l)
Autism [6][b] (non-chelated)	15 (2 – 29)	3 (1 – 43)	1.5 (0 – 23)	1 (1 – 5)	36[f] (10 – 1(
ASD[c] [19] (non-chelated)	12 (2 – 79)	2 (0 – 43)	1 (0 – 23)	1 (0 – 5)	32[g, h] (5 – 10
PDD-NOS or Asperger's Disorder [13] (non-chelated)	12 (2 – 79)	2 (0 – 6)	1 (0 – 16)	1 (0 – 3)	22 (5 – 6:
ASD [18] (chelated)[d]	11 (2 – 88)	1 (0 – 12)	0 (0 – 11)	1 (0 – 3)	19[h] (3 – 6:
Controls[e] [7]	7 (4 – 72)	2 (1 – 11)	0 (0 – 1)	1 (1 – 2)	16[f, g] (14 – 2

[a] Median urine porphyrin markers (range)

[b] Number of patients in the group examined

[c] Autism spectrum disorders (ASD), includes children diagnosed with autism, pervasive developmental delay – not otherwise specified (PDD-NOS), or Asperger's disorder.

[d] Patients past medical history indicated that they had previously received chelation treatment for > 3 months.

[e] Neurotypical siblings of children with ASD that were sex-, age-, and race-matched to the ASD population examined.

[f] Differences observed between the autism (non-chelated) and control group were significantly different ($p < 0.05$) based upon the non-parametric Mann-Whitney U test statistic (two-tailed *p*-value).

[g] Differences observed between the ASD (non-chelated) and control group were significantly different ($p < 0.05$) based upon the non-parametric Mann-Whitney U test statistic (two-tailed *p*-value).

[h] Differences observed between the ASD (non-chelated) and ASD (chelated) group were significantly different ($p < 0.05$) based upon the non-parametric Mann-Whitney *U* test statistic (two-tailed *p*-value).

those with autism having mean coproporphyrin levels (mean ± SD = 45.83 ± 32.28 µg/l) more than 2 SD above controls (mean ± SD = 16.14 ± 3.29 µg/l). It was also found that those with non-chelated ASDs had significantly increased (2-fold) median coproporphyrin levels in comparison to controls, with 58% of those with ASDs having coproporphyrin levels (mean ± SD = 34.32 ± 23.83 µg/l) more than 2 SD above controls (mean ± SD = 16.14 ± 3.29 µg/l). Finally, it was determined that those patients with unchelated pervasive developmental delay - not otherwise specified (PDD-NOS) or Asperger's disorder had a non-significantly increased (1.4-fold) median coproporphyrin levels in comparison to controls, with 46% of those with PDD-NOS or Asperger's disorder having levels (mean ± SD = 29.00 ± 17.91 µg/l) more than 2 SD above the controls (mean ± SD = 16.14 ± 3.29 µg/l).

Additionally, it was found that, among patients with ASDs, there was a significant increase (1.7-fold) in the median levels of coproporphyrin observed among those patients with non-chelated ASDs in comparison to those patients with chelated ASDs (these patients had been previously chelated for > 3 months). It was observed that patients with ASDs who had been chelated had non-significant differences in median levels of coproporphyrin compared with controls.

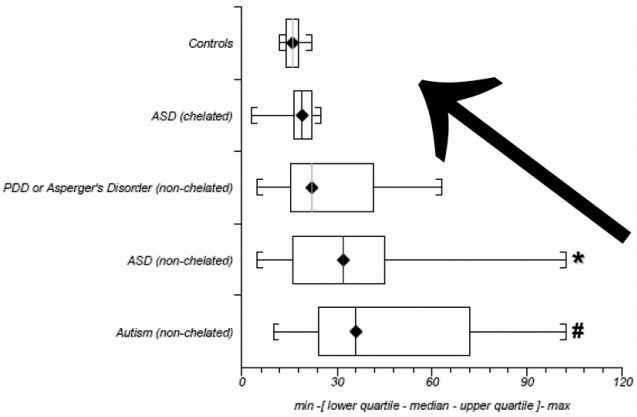

FIGURE 1 A summary of the apparent dose-response between median urine coproporphyrin levels (µg/l) and ASD clinical symptom severity observed in the present study. Autism spectrum disorders (ASD), includes children diagnosed with autism, pervasive developmental delay - not otherwise specified (PDD-NOS), or Asperger's disorder.

Chelated patients past medical history indicated that they had previously received chelation treatment for > 3 months. Controls were neurotypical siblings of children with ASD that were sex-, age-, and race-matched to the ASD population examined.

Differences observed between the autism (non-chelated) and control group were significantly different ($p < 0.05$) based upon the non-parametric Mann-Whitney *U* test statistic (two-tailed *p*-value).

* Differences observed between the ASD (non-chelated) and control group were significantly different ($p < 0.05$) based upon the non-parametric Mann-Whitney *U* test statistic (two-tailed *p*-value). Differences observed between the ASD (non-chelated) and ASD (chelated) group were significantly different ($p < 0.05$) based upon the non-parametric Mann-Whitney *U* test statistic (two-tailed *p*-value).

DISCUSSION

The results of the present study show an apparent dose-response association between ASD severity and porphyrinuria as summarized in figure 1.

Coproporphyrin levels were observed to be the most significantly elevated among non-chelated patients with autism in comparison to age-, sex-, and race-matched neurotypical ASD sibling controls. Autism is the most clinically severely affected of the ASDs examined in the present study. By contrast non-chelated patients with PDD-NOS or Asperger's disorder, which are significantly less clinically severely affected ASDs than autism, did not have significantly increased coproporphyrin levels in comparison to controls. It was found when evaluating non-chelated ASD patients as a group (including patients diagnosed with autism, PDD-NOS, or Asperger's disorder) that there was a significant increase in urinary coproporphryin levels in comparison to the controls employed, and that

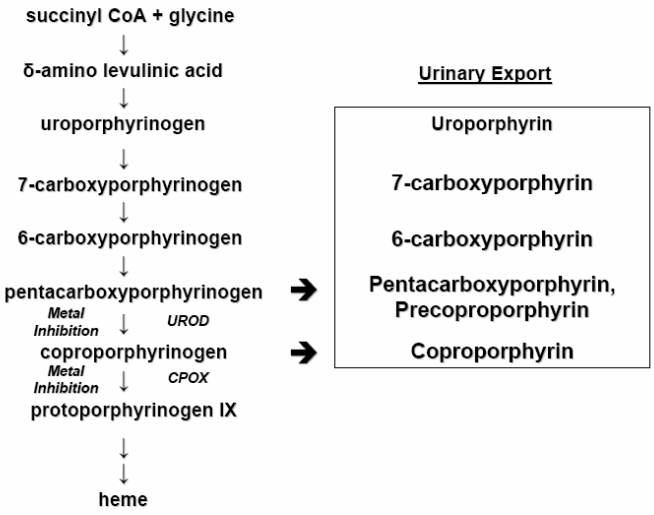

FIGURE 2 A summary of the heme synthesis pathway and major urinary metabolites. Porphyrinogens appear in urine as porphyrin derivates (right). Heavy metals can result in increased urinary pentaporphyrin, precoproporphyrin, and coproporphyrin by inhibiting uroporphyrinogen decarboxylase (UROD) and/or coproporphyrinogen oxidase (CPOX); urinary uroporphyrin is not reported to alter with inhibition of these enzymatic steps.

the elevation in urinary copropophyrin observed was between that observed for the autism group and the PDD-NOS/Asperger's disorder group. Finally, it was observed that the neurotypical sibling controls had the lowest levels of urinary coproporphyrin among any of the groups examined in the present study.

In evaluating the groups of patients examined for their urinary levels of coproporphyrin that were more than two SD above the mean of the neurotypical ASD sibling controls, it was found that the apparent dose-response relationship between ASD severity and urinary coproporphyrin levels persisted. It was found overall that 83% of non-chelated autism patients, 58% of non-chelated ASD patients, and only 46% of PDD-NOS/Asperger's disorder patients had urinary levels of coproporphyrin that were more than two SD above the mean of neurotypical ASD sibling controls.

It was also found that there was a significant increase in urinary levels of coproporphyin among non-chelated ASD patients in comparison to chelated (receiving chelation therapy > 3 months) ASD patients. Among the patients examined, it was determined that urinary levels of uroporphyrin, heptacarboxylporphyrin, hexacarboxylporphyrin, and pentacarboxylporphyrin were not significantly different.

The neurotypical ASD sibling controls utilized in the present study were selected, so as to minimize potential confounders, and may provide a very conservative estimate for potential background urinary porphyrin markers. The neurotypical ASD sibling controls were matched to the ASD patients for sex, age, and race. Additionally, they were selected to be siblings of ASD patients, so as to attempt to minimize unique household factors that might lead to increased porphyrin levels in ASD patients. The values observed for urinary porphyrin makers in the sibling controls employed in the present study were consistent with those observed in previous studies examining similarly aged children (mean coproporphyrin level = 11.4 μmol/mol creatinine or mean coproporphyrin level = 8.5 μmol/mol creatinine) (Nataf *et al.*, 2006), although the sibling controls (mean coproporphyrin level = 16.14 μg/l) examined in the present study had somewhat higher levels of coproporphyrin than those previously reported. This may indicate that ASD siblings share at least some common susceptibility/exposure factors with their affected siblings, and as a result, may have helped to minimize the associations observed between ASDs and urinary coproporphryin levels observed in the present study.

In considering the results from the present study, porphyrins are derivatives of the heme synthesis pathway that afford a measure of xenobiotic exposure (Brewster, 1988). Heme production primarily occurs in liver, kidney, and erythroid cells. The synthesis process occurs in two steps, from succinyl-CoA + glycine to uroporphyrinogen, and in a further series of steps, via pentacarboxyporphryinogen and coproporphyrinogen to heme, as summarized in figure 2 (Nataf *et al.*, 2006). Excess porphyrinogen metabolites are excreted in the urine as oxidized porphyrins, particularly uropophyrin and copropophyrin, reflecting the most abundant molecules in the kidney cortex and solubility (Woods and Miller, 1993): mid-pathway porphyrins are the most water-soluble and appear predominantly in urine, whereas hydrophobic protophyrin appears predominantly in bile and feces (Nataf *et al.*, 2006).

Excess urinary porphyrin excretion or porphyrinuria results from inhibition of key enzymatic steps in conditions including genetic deficiencies in heme production enzymes (Sarkany, 1999), hepatitis, renal, and erythroid disease (Gross *et al.*, 2000), and also by toxic inhibition of heme synthesis enzymes (Nataf *et al.*, 2006). In both experimental animals and humans exposed to heavy metals, porphyrins are exported at elevated levels into urine (Bowers *et al.*, 1992; Woods, 1996). The steps in heme pathway most vulnerable to heavy metal inhibition are uroporphyrin decarboxylase (UROD) (Woods and Kardish, 1983) and coproporphyrinogen oxidase (CPOX) reactions (Woods *et al.*, 2005), resulting in specific elevations of coprophyrin and pentacarboxyporphyrin in urine. A causal relationship between heavy metal inhibition and porphyrinuria has been demonstrated: both in rats exposed to mercury (Pingree *et al.*, 2001) and in humans exposed to lead (Rosen and Markowitz, 1993). It was observed in both cases that heavy metal removal with chelating agents reduced urinary porphyrin levels to control values. Although non-metal agents targeting the heme pathway can also elevate urinary porphyrin levels (Daniell *et al.*, 1997), precoproporphyrin (also known as keto-isocoproporphyrin) is produced by *in vivo* conversion of pentacarboxyporphyrinogen under pressure of heavy metal interference (Woods *et al.*, 2005; Heyer *et al.*, 2006), providing a specific porphyrin marker particular of mercury toxicity (Nataf *et al.*, 2006).

The results of the present study are in accord with those reported by Nataf *et al.* (2006) on a French cohort of autistic children. In both studies, it was observed that patients with autism had significantly increased mean urinary levels of coproporphyrin relative to controls (present study = 2.8-fold, Nataf *et al.* = 2.6-fold), and that > 50% of patients with autism had urinary levels of coproporphyrin more than two SD above controls. It was also found in both studies that other less severe conditions within the ASD categories, such as those diagnosed with PDD-NOS or Asperger's disorder,

tended to have less significant increases in urinary porphyrin levels relative to controls. Additionally, it was observed in both studies that chelation appeared to result in significant reductions in the levels of urinary coproporphyrin. The Nataf *et al.* study also increased the potential for an association between mercury exposure and some autistic disorders by finding a significant increase in precoproporphyrin levels in comparison to controls (Nataf *et al.*, 2006).

The results of the present study and those of Nataf *et al.* (2006), that support an association between heavy metal exposure, especially mercury exposure, and some autistic disorders, are buttressed by several other recent studies. Bradstreet *et al.* (2003) showed that, when comparing 221 children with ASDs to 18 age- and gender-matched neurotypical controls, following chelation therapy with *meso*-2,3-dimercaptosuccinic acid (DMSA), there were approximately 3-times significantly greater urinary mercury concentrations among autistics relative to controls, whereas autistics and controls had similar urinary concentrations of other heavy metals. Likewise, Holmes *et al.* (2003) examined first baby haircuts and determined that a group of 94 autistics had significantly higher body-burdens of mercury in comparison to 45 age- and gender-matched non-autistic controls by demonstrating that the ability to excrete mercury in first baby haircuts was inversely proportional to the severity of autistics. On the whole, the ability of autistics to excrete mercury was very low compared to non-autistic matched controls.

In addition, it has been reported that the neurotoxicity of mercury is associated with depletion of glutathione. Mercury binds to cysteine thiol (-SH) groups on intracellular proteins and inactivates their function. The cysteine-SH group of glutathione binds mercury and protects essential proteins from functional inactivation. Glutathione is the major mechanism of mercury excretion, and individuals with genetic deficiencies in glutathione synthesis are less able to excrete mercury, making them more sensitive to its adverse effects (James *et al.*, 2005). James *et al.* (2004) have shown that 20 autistic children in comparison to 33 age- and gender-matched non-autistic control children had significant reductions in total glutathione (46%). Additional studies on a larger cohort of almost 100 autistic children in comparison to almost 50 healthy non-autistic controls have appeared to confirm the previous work of James *et al.* (2004), and have revealed a signature transsulfuration metabolic imbalance that is present in many autistic children, suggesting that these children would be susceptible to the harmful effects of mercury exposure (Environmental Working Group, 2004). Several recent studies have also identified markers of mercury-mediated oxidative stress in some ASDs (McGinnis, 2004; Chauhan and Chauhan, 2006). Furthermore, recent epidemiological studies have associated genomic susceptibility factors in mercury detoxification pathways with some ASDs (Boris *et al.*, 2004; Serajee *et al.*, 2004; Buyske *et al.*, 2006).

CONCLUSION

This study provides the first clinical evidence from Americans with ASDs that associates them with specific urinary porphyrin markers known to be associated with heavy metals. The results of the present study are consistent with those observed in a large cohort of French children with ASDs.

This study is also the first to utilize readily commercially available clinical laboratory urinary porphyrin testing (LabCorp, Inc.) to associate specific urinary porphyrin markers with ASDs. The testing employed in the present study is non-invasive, and readily practical to utilize in a clinical setting. It is important when evaluating urinary porphyrin samples that age- and sex-adjusted reference ranges be examined because these factors may influence urinary porphyrin levels (Nataf *et al.*, 2006).

The results from the present study suggest that children with ASDs should be routinely clinically evaluated for urinary porphyrin markers, and that treatment protocols on ASD patients should consider monitoring urinary porphyrin markers. Furthermore, the results of the present study provide insights into the apparent dose-response effect mercury exposure may have in some children with ASDs, and suggest that additional research should be conducted to evaluate mercury exposure in ASDs.

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Potential Conflict of Interest:

Dr. Mark R. Geier has been an expert witness and a consultant in vaccine/biologic cases before the no-fault National Vaccine Injury Compensation Program (NVICP) and in civil litigation. David Geier has been a consultant in vaccine/biologic cases before the no-fault NVICP and in civil litigation.

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