A Prospective Study of Mercury Toxicity Biomarkers in Autistic Spectrum Disorders

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Porphyrrins are derivatives formed in the heme synthesis pathway and porphyrins afford a measure of xenobiotic exposure. The steps in the heme pathway most vulnerable to heavy metal inhibition are uroporphyrin decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX) reactions. Mercury toxicity was associated with elevations in urinary coproporphyrin (cP), pentacarboxyporphyrin (5cxP), and precoproporphyrin (prcP) (also known as keto-isocoproproporphyrin) levels. Two cohorts of autistic patients in the United States and France had urine porphyrin levels associated with mercury toxicity. A prospective study of urinary porphyrin testing at LabCorp (United States) and the Laboratoire Philippe Auguste (France) involving 71 autism spectrum disorder (ASD) patients, neurotypical sibling controls, and general population controls was undertaken. ASD patients had significant elevations in urinary levels of cP, 5cxP, and prcP relative to controls, and > 50% of ASD patients had urinary cP levels more than 2 standard deviations above the mean values for neurotypical sibling controls. Significant reductions in urinary 5cxP and cP levels were observed in ASD patients following chelation. A significant correlation was found between urinary porphyrins measured at LabCorp and those measured at the Laboratoire Philippe Auguste on individual ASD patients. The established developmental neurotoxicity attributed to mercury and biochemical/genomic evidence for mercury susceptibility/toxicity in ASDs indicates a causal role for mercury. Urinary porphyrin testing is clinically available, relatively inexpensive, and noninvasive. Porphyrins need to be routinely measured in ASDs to establish if mercury toxicity is a causative factor and to evaluate the effectiveness of chelation therapy.

Porphyrrins are derivatives of the heme synthesis pathway that afford a measure of xenobiotic exposure (Brewster, 1988). Heme production primarily occurs in liver, kidneys, and erythroid cells. The synthetic process is summarized in Figure 1 (Nataf et al., 2006). Excess porphyrinogen metabolites are excreted in the urine as oxidized porphyrins, particularly uroporphyrin (uP) and coproporphyrin (cP), the most abundant soluble porphyrin molecules in the kidney cortex (Woods & Miller, 1993). Because these mid-pathway porphyrins are the most water-soluble of all the porphyrins, they are excreted predominantly in urine, whereas the hydrophobic protoporphyrin is predominantly found in the bile and feces.

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 disease, and erythroid disease (Gross et al., 2000), as well as by heavy metal inhibition of heme enzyme synthesis (Woods, 1996). Both in experimental animals and in humans exposed to heavy metals, elevated levels of porphyrins are found in the urine (Bowers et al., 1992; Woods, 1996). The steps in the heme pathway most vulnerable to heavy metal inhibition are those in which uroporphyrin decarboxylase (UROD) (Woods & Kardish, 1983) and coproporphyrinogen oxidase (CPOX) (Woods et al., 2005) are involved. The result of these inhibitions is specific elevations of cP and pentacarboxyporphyrin (5cxP) in the urine. A causal relationship between heavy metal inhibition and porphyrinuria was demonstrated both in rats (Pingree et al., 2001) and humans exposed to mercury (Woods et al., 1993), as well as in humans exposed to lead (Rosen & Markowitz, 1993). Investigators also observed that heavy metal removal with chelating agents reduced urinary porphyrin levels to control values (Gonzalez-Ramirez et al., 1995). Although nonmetal agents
targeting the heme pathway also elevate urinary porphyrin levels (Daniell et al., 1997), precoproporphyrin (prcP) (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, in particular, a specific porphyrin marker for mercury exposure (Woods, 1995).

Recently, a cohort of autistic patients from France (Nataf et al., 2006) and a cohort of autism spectrum disorder (ASD) patients from the United States (Geier and Geier, 2006d) exhibited urinary porphyrin patterns indicative of mercury toxicity. Following utilization of two independent labs (France, Laboratoire Philippe Auguste, and United States, LabCorp), lab results showed that autistic and ASD patients had two- to threefold significantly increased urinary levels of 5cxP, prcP, and cP relative to controls. Furthermore, it was observed that chelation therapy among autistic and ASD patients significantly lowered urinary levels of cP and prcP.

The purpose of the present study was to conduct an expanded assessment of urinary porphyrin levels among ASD patients, and to compare and contrast urinary porphyrin patterns reported by two clinical labs, Laboratoire Philippe Auguste and LabCorp, on an expanded cohort of U.S. ASD patients.

MATERIALS AND METHODS

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

Subjects

Study subjects were consecutive patients with ASDs who prospectively presented to the Genetic Centers of America for outpatient genetic evaluations from 2005 to 2006. Each patient was previously diagnosed by a physician with an ASD based upon the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM–IV) criteria. In total, 71 patients with ASDs were identified. Table 1 summarizes the overall profile of the patients with ASDs examined in the present study. Each patient’s medical record was reviewed to assess chelation status. Patients were considered unchelated if they had never been chelated. Chelated patients were those who had received chelation therapy (e.g., meso-2,3-dimercaptosuccinic acid [DMSA] or 2,3-dimercapto-1-propanesulfonic acid [DMPS]) that lasted longer than 3 mo and who had their chelation therapy documented in their medical records. Each patient was also tested to exclude those with brain structural abnormalities (computed tomography [CT] or magnetic resonance imaging [MRI] head scans) from the present study. In addition, lab 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, Prader–Willi syndrome/Angelman, urine organic acid abnormalities, and Rett syndrome (LabCorp).

Evaluation

In total, 63 ASD patients were tested by the CLIA-certified LabCorp for urine porphyrin markers (urine porphyrin analysis was conducted using high-pressure liquid chromatography with fluorometric detection), including uP, heptacarboxyporphyrin (7cxP), hexacarboxyporphyrin (6cxP), 5cxP, and cP (type I) (Ford et al., 1981). Additionally, 23 ASD patients were also tested by the ISO-certified Laboratoire Philippe Auguste for urine porphyrin markers (urine porphyrin analysis was conducted using high-pressure liquid chromatography with fluorometric detection), including uP, 7cxP, 6cxP, 5cxP, prcP, and cP (types I and III) (Bowers et al., 1992).

Controls

Age-, gender-, and race-matched neurotypical siblings of ASD patients were used as controls. Table 1 summarizes the overall profile of the sibling controls examined in the present study. Each sibling control was tested by LabCorp for urine porphyrin markers, including uP, 7cxP, 6cxP, 5cxP, and cP. In addition, consecutive patients without neurodevelopmental disorders were also used as general population controls. Table 1 summarizes the overall profile of the general population controls examined in the present study. Each general population control was tested by Laboratoire Philippe Auguste for urine porphyrin markers, including uP, 7cxP, 6cxP, prcP, and cP.
Lab Specimens

All the samples tested for porphyrins were light-protected and from first morning urine collections. The samples tested by the two labs were from different urine collections.

Statistical Analyses

In the present study, urinary porphyrin levels were evaluated among unchelated ASD patients in comparison to age-, gender-, and race-matched sibling controls (LabCorp testing). The urinary porphyrin levels were examined among unchelated ASD patients in comparison to chelated ASD patients (LabCorp testing). Since urinary porphyrin levels were similar among chelated ASD patients and matched sibling controls, urinary prophyrin levels were also compared among unchelated ASD patients in comparison to the composite results for chelated ASD patients and matched sibling controls (LabCorp testing). Finally, urinary porphyrin levels were compared among unchelated ASD patients in comparison to general population controls (Laboratoire Philippe Auguste testing). The null hypothesis for each of these comparisons was that the groups examined should have similar urinary porphyrin levels. The nonparametric Mann–Whitney U-test statistic was utilized to determine statistical significance for each of these statistical tests.

In addition, the plotted interrelationship between urinary cP levels measured at LabCorp (µg cP/L) and the results reported by Laboratoire Philippe Auguste (nmol cP/nmol uP) was evaluated for some individual ASD patients. The null hypothesis was that the slope of the line would be equal to zero. The simple linear regression test statistic was utilized to determine statistical significance.

For all statistical tests in the present study, a two-tailed p value ≤0.05 was considered statistically significant, and the statistical package contained in StatsDirect (Version 2.4.2) was employed.

RESULTS

Table 2 summarizes the overall urinary porphyrin results from the LabCorp testing examined in the present study. The mean urinary 5cXP levels reported were significantly elevated among unchelated ASD patients in comparison to chelated ASD patients (ratio = 1.6) and to the chelated ASD patients plus the sibling controls (ratio = 1.6). Additionally, it was found that mean urinary cP levels were significantly increased among unchelated ASD patients in comparison to the chelated ASD patients (ratio = 1.8) and to the sibling controls (ratio = 1.9), and to the chelated ASD patients plus the sibling controls (ratio = 1.8). It was also observed that when comparing the
TABLE 2
A Summary of the Overall Urinary Porphyrin Data From LabCorp Testing Examined in This Study

<table>
<thead>
<tr>
<th>Population examined</th>
<th>uP$^a$ (µg/L)</th>
<th>7cxP (µg/L)</th>
<th>6cxP (µg/L)</th>
<th>5cxP (µg/L)</th>
<th>cP (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchelated ASD ($n = 37$)</td>
<td>28.51 ± 38.37</td>
<td>3.7 ± 7.16</td>
<td>1.97 ± 4.56</td>
<td>1.57 ± 1.04$^b$</td>
<td>29.97 ± 19.1$^{d,e}$</td>
</tr>
<tr>
<td>Chelated$^d$ ASD ($n = 26$)</td>
<td>15.54 ± 17.67</td>
<td>2.54 ± 2.85</td>
<td>1.08 ± 2.67</td>
<td>1 ± 0.85</td>
<td>16.46 ± 9.51</td>
</tr>
<tr>
<td>Sibling controls ($n = 9$)</td>
<td>16.89 ± 21.6</td>
<td>2.89 ± 3.18</td>
<td>0.44 ± 0.53</td>
<td>1 ± 0.5</td>
<td>16.22 ± 4.32</td>
</tr>
<tr>
<td>Chelated ASD + sibling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls ($n = 35$)</td>
<td>15.89 ± 18.43</td>
<td>2.63 ± 2.89</td>
<td>0.91 ± 2.32</td>
<td>1 ± 0.77</td>
<td>16.4 ± 8.42</td>
</tr>
</tbody>
</table>

Note. The nonparametric Mann–Whitney U-test statistic (two-sided $p$ value) was employed; uP = uroporphyrin; 7cxP = heptacarboxyporphyrin; 6cxP = hexacarboxyporphyrin; 5cxP = pentacarboxyporphyrin; cP = coproporphyrin.

$^a$Mean ± standard deviation.
$^b$There was a significant difference between the unchelated ASD patients in comparison to chelated ASD patients ($p < .05$).
$^d$There was a significant difference between the unchelated ASD patients in comparison to the general population controls ($p < .05$).
$^e$There was a significant difference between the unchelated ASD patients in comparison to the sibling controls ($p < .05$).
$^f$There was a significant difference between the unchelated ASD patients in comparison to chelated ASD patients ($p < .05$).
$^g$Patients past medical history indicated that they had previously received chelation treatment for > 3 months prior to the collection of urine sample.

TABLE 3
Summary of the Overall Urinary Porphyrin Data From Laboratoire Philippe Auguste Testing Examined in This Study

<table>
<thead>
<tr>
<th>Population examined</th>
<th>7cxP/uP$^a$ (nmol/nmol)</th>
<th>prcP/uP (nmol/nmol)</th>
<th>cP/uP (nmol/nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unchelated ASD ($n = 11$)</td>
<td>0.22 ± 0.10</td>
<td>1.67 ± 2.8$^b$</td>
<td>26.66 ± 49.19$^e$</td>
</tr>
<tr>
<td>General population controls ($n = 5$)</td>
<td>0.17 ± 0.03</td>
<td>0.36 ± 0.09</td>
<td>5.56 ± 1.48</td>
</tr>
<tr>
<td>Laboratory reference range</td>
<td>(0.17–0.25)</td>
<td>(0.3–0.6)</td>
<td>(5.55–7.14)</td>
</tr>
</tbody>
</table>

Note. The nonparametric Mann–Whitney U-test statistic (two-sided $p$ value) was employed; uP = uroporphyrin; 7cxP = heptacarboxyporphyrin; prcP = precoproporphyrin; cP = coproporphyrin.

$^a$Mean ± standard deviation.
$^b$There was a significant difference between the unchelated ASD patients in comparison to the general controls ($p < .05$).
$^e$There was a significant difference between the unchelated ASD patients in comparison to the general population controls ($p < .05$).
in the ASD patients examined. Second, as controls, the urines from age-, gender-, and race-matched neurotypical siblings were examined. The neurotypical ASD sibling controls utilized were selected to minimize potential confounders and to provide a conservative estimate for potential background urinary porphyrin markers. They were selected so as to minimize unique household factors that might be associated with increased porphyrin levels in ASD patients. The values observed for urinary porphyrin markers in the sibling controls employed in the present study were consistent with those observed in previous studies examining similarly aged children (Nataf et al., 2006), although the sibling controls examined in the present study had somewhat higher levels of cP than those previously reported. This may indicate that non-ASD siblings share at least some common susceptibility/exposure factors with their affected ASD siblings, and, as a result, may have helped to minimize the magnitude of the associations observed between ASDs and urinary 5cxP and cP levels observed in the present study. In addition to sibling controls, ASD patients who had received chelation therapy for more than 3 mo were also utilized as controls. It was observed that ASD patients who received chelation therapy for more than 3 mo had urinary porphyrin levels similar to those of sibling controls, and hence consistent quantitative increased urinary 5cxP and cP levels similar to those observed for sibling controls. This consistency of observation helps to minimize the possibility that the present results were simply the result of chance or confounding. Third, two distinct methods of reporting urinary porphyrin levels were used by two separate labs, which further minimizes the effects of chance or confounding. Both laboratories analyzing urinary porphyrin samples in the present study found that the urinary porphyrin metabolites associated with mercury toxicity (i.e., 5cxP, prcP, and cP) were significantly elevated in unchelated ASD patients in comparison to controls, whereas other urinary porphyrin metabolites, not associated with mercury toxicity (i.e., uP, 7cxP, and 6cxP), were measured at similar levels in unchelated ASD patients, chelated ASD patients, and controls. Finally, the nonparametric Mann–Whitney U-test statistic was employed for statistical testing in the present study. Because the nonparametric Mann–Whitney U-test statistic makes minimal assumptions regarding the distribution of the data examined, it provides conservative statistical estimates. Additionally, a twosided p-value of $\leq .05$ was considered statistically significant.

In considering the results of the present study, the urinary porphyrin patterns observed among the ASD patients tested are consistent with previous observations reported in cohorts of ASD patients in the United States (Geier & Geier, 2006d) and autism patients in France (Nataf et al., 2006). Notably, all three studies reported that autism or ASD patients had significantly increased urinary levels of 5cxP, prcP, and/or cP in comparison to controls. Moreover, all three studies found that $>50\%$ of the unchelated autistic or ASD patients had urinary cP levels more than 2 standard deviations above the control mean. Additionally, all three studies reported that chelation therapy given to the mercury-intoxicated autism or ASD patients resulted in significant reductions in urinary porphyrin levels associated with increased body burdens of mercury.

In addition to previous studies examining urinary porphyrin patterns in ASD patients, a series of other studies attempted to assess the body burden of heavy metals in ASDs. Bradstreet et al. (2003) examined urinary heavy metal concentrations following therapy for 3 days with DMSA, a U.S. Food and Drug Administration (FDA)-approved chelating agent, in children with ASDs in comparison to matched controls. Overall, urinary mercury concentrations were about threefold higher in children with ASDs than in neurotypical controls. In contrast, similar urinary cadmium and lead concentrations were observed between children with ASDs and those used as controls.

A case series of ASD patients who had normal development for the first year of life and subsequently, following significant exposure to medicinal sources of mercury, underwent regressions into autistic disorders between their first and second birthdays was evaluated (Geier & Geier, 2007a). It was observed that, following chelation therapy, each of these ASD patients presented with significantly elevated concentrations of mercury in their urine, feces, and/or hair samples. Additionally, it was observed in two clinical trials that chelation therapy, in addition to significantly increasing urinary concentrations of heavy metals (including mercury), resulted in significant neuropsychological testing improvements in ASD patients (Geier & Geier, 2006b; Lonsdale et al., 2002).

Adams et al. (2007) evaluated baby teeth as a measure of cumulative exposure to toxic metals during fetal development and early infancy in autistic children relative to matched controls. The reported mean mercury level in baby teeth from autistic children ($\text{mean} = 0.15 \pm 0.11 \text{ppm}$) was 2.1-fold higher than the mean for the controls ($\text{mean} = 0.07 \pm 0.06 \text{ppm}$),
whereas lead and zinc levels were similar in both groups. These investigators concluded that, on mean, autistic children had a higher body burden of mercury during fetal/infant development than neurotypical children.

Figure 3 summarizes the apparent overall relationship between urinary porphyrins and mercury body burden in ASDs. The recorded ASD urinary porphyrin concentrations were reported to vary quantitatively with pre- and postchelation mercury body burden and to have an inverse correlation with urinary mercury content.

In previous studies, ASD patients were observed to show biochemical and genomic susceptibilities to mercury toxicity. The neurotoxicity of mercury is associated with depletion of glutathione. Mercury binds to cysteine thiol (-SH) groups on intracellular proteins and inactivates their functions. The cysteine -SH group of glutathione binds mercury and protects essential proteins from functional inactivation. Glutathione is the major mechanism for mercury excretion. 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). Holmes et al. (2003) reported that autistic children had on mean significantly higher in utero exposure to mercury but showed significantly decreased mercury levels in first baby haircut samples than did matched normal controls. This paradoxical result only may be explained by differential rates of pre- and postnatal mercury elimination in autistic and normal children. Furthermore, it was observed that, within the autistic group, mercury levels in the hair samples tested varied significantly across mildly, moderately, and severely autistic children, with mean group levels of 0.79, 0.46, and 0.21 ppm, respectively. In contrast, the mercury levels in the hair samples from the controls correlated significantly with sources of mercury exposure, including the number of the maternal amalgam fillings during pregnancy, as well as with exposure to mercury from Rho(D) immune globulins administered during pregnancy. These correlations were completely absent in the autistic group.

A series of studies revealed a signature transsulfuration metabolic imbalance present in many autistic children, characterized by significant reductions in blood cysteine, sulfate, total glutathione, and reduced glutathione levels relative to controls, which may be the result of increased mercury exposure (pre- and postnatal) and also would make ASD patients particularly susceptible to the harmful effects of mercury exposure (Environmental Working Group, 2004; Geier & Geier, 2006a; James et al., 2004; 2006; Waring et al., 1997). Additionally, Walker et al. (2006) showed that cultured lymphocytes from ASD patients, when challenged with a zinc compound, responded by a marked upregulation of metallothioneins (at least nine different metallothioneins were overexpressed),
while such cells when challenged with an organic mercury compound responded by upregulating numerous heat-shock proteins but, significantly, not metallothioneins.

Several recent studies also associated mercury exposure with ASDs (including identifying markers of mercury-mediated oxidative stress in ASDs; Chauhan & Chauhan, 2006; Kern & Jones 2006; Maya & Luna, 2006; McGinnis, 2004). Furthermore, recent epidemiological studies associated genomic susceptibility factors in mercury detoxification pathways with ASDs (Boris et al., 2004; Buyske et al., 2006; James et al., 2006; Seraje et al., 2004).

Mercury exposure was also observed to be significantly associated with ASDs in a series of epidemiological studies (Counter et al., 2002; Geier & Geier, 2005, 2006c, 2007b; Holmes et al., 2003; Palmer et al., 2006; Rury, 2006; Windham et al., 2006). In the most recent epidemiological study published from California (supported by the Centers for Disease Control and Prevention), 283 children with ASDs and 657 controls, born in 1994 in the San Francisco Bay area, were examined (Windham et al., 2006). These researchers assigned exposure level by census tract of birth residence for 19 chemicals. Among the 19 chemicals examined to which children were exposed, mercury was found to be the single largest risk factor associated with autistic disorders. When comparing high mercury relative to low mercury exposure, there was a statistically significant increase in risk, which was about double, for having an autistic disorder.

Faustman et al. (2000) 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)” (p. 15). Investigators have also reported that exposure to mercury produced 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 (Bernard et al., 2001, 2002; Blaxill et al., 2004; Mutter et al., 2005; Redwood et al., 2001; Zahir et al., 2005). Additionally, Hornig et al. (2004) observed features reminiscent of autism in a susceptible mouse strain following Thimerosal exposure through vaccines. The symptoms observed included growth delay, reduced locomotion, exaggerated response to novelty, increased brain size, decreased numbers of Purkinje cells, significant abnormalities in brain architecture affecting areas subserving emotion and cognition, and densely packed hyperchromic hippocampal neurons with altered glutamate receptors and transporters.

CONCLUSION

Taken together, the consistency of the results observed in the present study between the urinary porphyrin levels measured at both LabCorp and the Laboratoire Philippe Auguste and their agreement with observations made in previous examinations of autism and ASD patients in France and the United States indicate that many ASD patients have urinary porphyrin profiles consistent with significantly increased body burdens of mercury. Additionally, a number of other studies have correlated an increased body burden of mercury in ASD patients with: (1) increased pre- and postnatal mercury exposure, (2) significant increases in the mean mercury level in the baby teeth, (3) significant elevations in urinary mercury concentrations following chelation therapy, and (4) a significant decrease in the rate of mercury excretion documented in first baby haircuts. Furthermore, given the established developmental neurotoxicity attributed to mercury and the established biochemical and genomic susceptibility factors to mercury toxicity in ASDs, clearly, mercury exposure may play a causal role in a significant number of ASDs. Additional research needs to be conducted to further evaluate mercury toxicity in ASDs. Since urinary porphyrin testing is clinically available, relatively inexpensive, and noninvasive, porphyrin levels, especially 5cP, prcP, and prcP, should be routinely measured in ASD patients; these represent the kidney mercury content and may assess the patient’s mercury body burden.

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