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A Prospective Blinded Evaluation of Urinary Porphyrins Verses the Clinical Severity of Autism Spectrum Disorders

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A prospective, blinded study evaluated the relationship between autism spectrum disorder (ASD) severity measured by Childhood Autism Rating Scale (CARS) scores and urinary porphyrins among a cohort of participants ($n = 26$). LabCorp (CLIA-approved) tested for uroporphyrins, heptacarboxylporphyrins, hexacarboxylporphyrins, pentacarboxylporphyrins, coproporphyrin (cP) I, and cP III levels. Participants with severe ASD had significantly increased cP I, cP III, and total cP levels in comparison to participants with mild ASD. A significant correlation was observed between increasing cP levels and CARS scores. Significant correlations were also noted for comparative urinary porphyrin testing between LabCorp and the Laboratoire Philippe Auguste (ISO-approved) for total cP. Finally, total cP measured at LabCorp was found to significantly correlate with precoproporphryin (a specific porphyrin marker for mercury toxicity) measured at the Laboratoire Philippe Auguste. Since urinary porphyrin testing is clinically available, relatively inexpensive, and noninvasive, it may be used to help suggest whether heavy metal toxicity is associated with ASD.

Nataf et al. (2006) were the first investigators to describe elevations in specific urinary porphyrin metabolites in a cohort of subjects diagnosed with autism spectrum disorders (ASD). These investigators observed that urinary porphyrins,

pentacarboxyporphyrin (5cxP), precoproporphyrin (prcP), and total coproporphyrins (cP) (I + III), which are associated with increased mercury (Hg) body burden, were significantly elevated in subjects diagnosed with autism ($n = 106$) relative to controls. In contrast, other urinary porphyrin metabolite levels were similar among subjects diagnosed with autism in comparison to controls. Further, these investigators observed that 5cxP, prcP, and total cP (I + III) were reported to increase across the ASD spectrum from mild to severe clinical symptoms (Asperger's disorder < pervasive developmental delay–not otherwise specified (PDD-NOS) < autism < autism + epilepsy), whereas other urinary porphyrin metabolites were not found to significantly fluctuate in correspondence with ASD severity. Finally, these investigators observed that *meso*-2,3-dimercaptosuccinic acid (DMSA)-based chelation therapy significantly decreased urinary prcP and total cP (I + III) levels in a cohort of subjects diagnosed with autism.

Subsequent studies on cohorts of subjects diagnosed with ASD in the United States by Geier and Geier (2006, 2007b), in France by Nataf et al. (2008), and in Australia by Austin and Shandley (2008) have revealed comparable results. In addition, another recent study by Geier et al. (2009) evaluated urinary porphyrin metabolites in a prospective, blinded cohort study of subjects diagnosed with ASD. The study evaluated ASD severity based upon Childhood Autism Rating Scale (CARS) scores calculated prior to blind laboratory testing for urinary porphyrins. It was observed that study subjects with a severe ASD diagnosis in comparison to study subjects with a mild ASD diagnosis had significantly increased urinary porphyrin levels of 5cxP, prcP, and total cP (I + III), whereas other urinary porphyrin levels were similar in both groups. In addition, regression analyses showed significant relationships between increasing CARS scores and rising urinary 5cxP and prcP levels. These correlations were absent for other urinary porphyrin metabolites examined. Finally, it was observed that increasing urinary 5cxP and prcP levels were significantly correlated with impaired glutathione detoxification (Geier et al., 2009).

Porphyrins are derivatives of the heme synthesis pathway that afford a measure of xenobiotic exposure (Brewster, 1988). The steps in the heme pathway most vulnerable to heavy metal inhibition are those in which uroporphyrin decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX) are involved (Woods & Kardish, 1983; Woods et al., 2005). The result of these inhibitions is specific elevations of cP in the urine. A significant relationship between heavy metal inhibition of heme synthesis and porphyrinuria was demonstrated both in rats (Pingree et al., 2001) and in humans exposed to Hg (Woods et al., 1993), as well as in humans exposed to lead (Pb) (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), 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 Hg body burden (Woods, 1995).

The purpose of the present prospective, blinded study was to evaluate environmental toxicity in a cohort of participants diagnosed with ASD utilizing clinically available laboratory testing from the Laboratory Corporation of America (LabCorp) for urinary porphyrins. The aims of the study were to: (1) determine whether the clinical severity of ASD correlated with urinary porphyrin levels; (2) for those urinary porphyrins that were found to significantly correlate with the severity of ASD, examine the consistency of the measurements of such porphyrins at LabCorp with those measured at a second laboratory, the Laboratoire Philippe Auguste; and (3) for those urinary porphyrins that were found to significantly correlate with the severity of ASDs, examine the correlation of such porphyrins measured at LabCorp with prcP (a specific marker of Hg exposure) measured at the Laboratoire Philippe Auguste.

MATERIALS AND METHODS

The study was conducted at the Autism Treatment Center (Dallas, TX). Phlebotomy took place at Medical Center Plano, Outpatient Phlebotomy (Plano, TX). The study protocol received Institutional Review Board (IRB) approval from Liberty IRB, Inc. (Deland, FL). This IRB was utilized because the Autism Treatment Center routinely uses this IRB to approve its studies. 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.

Participants

The present study examined consecutive qualifying participants (n = 26) 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 13 yr of age and had an initial Childhood Autism Rating Scale (CARS) score ≥30 (Schopler et al., 1980). 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.

Clinical Evaluation

At the time of initial intake, 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 for each child. A CARS evaluation was performed by Dr. Kern, who interviewed the parents and observed each child. Dr. Kern is trained in the use of the CARS and has 12 yr of 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.

Laboratory Evaluation

Following the initial intake evaluation, each participant in the present study had 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. Urine samples analyzed by LabCorp were taken to and processed at LabCorp in Medical City Hospital (Dallas, TX). Separately, urine samples were also shipped to the Laboratiore Philippe Auguste (Paris, France). LabCorp and Laboratoire Philippe Auguste urine specimens were collected according to the respective laboratory protocols. The laboratoies used in the present study were blinded and received no information regarding the clinical status of the participants examined or their CARS scores prior to their testing of each sample.

Participants were tested for the following: (1) urinary porphyrin tests at LabCorp (all Clinical Laboratory Improvement Act/Amendment (CLIA)-approved) for uroporphyrin (uP), heptacarboxylporphyrins (7cxP), hexacarboxylporphyrins (6cxP), pentacarboxyporphyrins (5cxP), cP I, cP III; and (2) urinary porphyrin tests at Laboratoire Philippe Auguste, Paris, France (all International Organization for Standardization (ISO)-approved) for uP, 7cxP, 6cxP, 5cxP, prcP, and cP. The analytical testing methods utilized by the laboratories in the current study were previously described (Nataf et al., 2006).

Urinary porphyrins at LabCorp were measured in micrograms of the specific urinary porphyrin per liter of total urine volume. In order to adjust for potential variations in urinary volume and/or differences in porphyrin synthesis rates, 7cxP, 6cxP, 5cxP, cP I, and cP III concentrations were normalized to uP levels.

TABLE 1
A Summary of the Participants With an ASD Diagnosis Examined

Descriptive information	Overall (*n* = 26)	Mild ASD[a] (*n* = 13)	Severe ASD[b] (*n* = 13)
Gender/age			
Male/female (ratio)	23/3 (7.7:1)	12/1 (12:1)	11/2 (5.5:1)
Mean age in years ± SD (range)	5.7 ± 2.8 (2–13)	6.2 ± 3.3 (3–13)	5.2 ± 2.2 (2–9)
Race (*n*)			
Caucasian	69% (18)	85% (11)	54% (7)
Minorities[c]	31% (8)	15% (2)	46% (6)
Autistic disorder characteristics			
Mean CARS Score ± SD (range)	39 ± 6.6 (30–51)	33.6 ± 3.5 (30–38.5)	44.4 ± 3.8 (40–51)
Regressive (*n*)[d]	61% (16)	62% (8)	62% (8)
Nonregressive (*n*)	38% (10)	38% (5)	38% (5)
Autism (*n*)	69% (18)	62% (8)	77% (10)
Autism spectrum disorders (*n*)[e]	31% (8)	38% (5)	23% (3)
Previous treatments			
Supplements (*n*)	19% (5)	7.7% (1)	31% (4)

Note. All participants examined in the present study were living in the state of Texas and had not previously received chelation therapy.
[a]Mild ASD is defined as any study participant with a CARS score less than the overall study participant median (CARS score <39.25).
[b]Severe ASD is defined as any study participant with a CARS score greater than the overall study participant median (CARS score >39.25).
[c]Includes participants of Hispanic, Black, Asian, or mixed ancestry.
[d]Includes participants that had a regressive event in development at any time following birth.
[e]Autism spectrum disorders include participants diagnosed with pervasive developmental disorder–not otherwise specified (PDD-NOS) and Asperger's disorder.

Statistical Analyses

The current study used the statistical package contained in StatsDirect (Version 2.4.2). Urinary porphyrins measured at LabCorp 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 nonparametric Mann–Whitney *U*-test statistic. This division of data was undertaken because of the size of the sample examined, so as to ensure equal numbers of study participants in each group, as well as to provide adequate statistical power for discerning statistically significant differences between the groups studied. The null hypothesis stated that there should be no difference between the median for each urinary porphyrin between participants with mild and severe ASD. The nonparametric 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. In addition, for those porphyrins that were found to significantly correlate with ASD severity (i.e., cP levels), the nonparametric linear regression test statistic was utilized to evaluate the relationship between urinary porphyrin levels at LabCorp and those at Laboratoire Philippe Auguste. Finally, the cP levels measured at LabCorp were evaluated in comparison to prcP levels measured at the Laboratoire Philippe Auguste. The reason for this comparison was that LabCorp does not report prcP levels, and it was of interest to determine the potential for a correlation between cP levels and a more specific biomarker of elevated Hg body burden (i.e., prcP). The null hypothesis stated that the slope of the line would be equal to zero for the relationship between urinary porphyrin levels at LabCorp with Laboratoire Philippe Auguste.

For all statistical tests in the present study, nonparametric testing was utilized to minimize potential assumption of normality for the data distributions examined, as well as to minimize the potential effects of outliers in the data examined. Further, a two-tailed *p* value ≤.05 was considered statistically significant for all statistical tests.

RESULTS

Table 2 lists the urinary porphyrin levels among the participants with mild ASD in comparison to participants with severe ASD, defined as a CARS score below or above the median (39.25), respectively. It was observed that there were significant increases in the median urinary levels of cP III (µg/L) (4.5-fold) and total cP (I + III) (µg/L) (3.1-fold) among the participants with severe ASD in comparison to participants with mild ASD. In addition, the median ratios of cP I/uP (1.5-fold), cP III/uP (3.3-fold), and total cP (I + III)/uP (2.9-fold) were

TABLE 2
An Assessment of Urinary Porphyrin Levels Among the Participants With a Mild ASD[a] in Comparison
to Participants With a Severe ASD[b]

Laboratory test [reference range]	Mild ASD cases ($n = 13$), median [IQR]	Severe ASD cases ($n = 13$), median [IQR]
μg/L:		
Uroporphyrins [0–20]	9 [4–13]	7 [4–8]
Heptacarboxyporphyrins [0–2]	1 [0–2]	1 [1–1]
Hexacarboxyporphyrins [0–1]	0 [0–0]	0 [0–0]
Pentacarboxyporphyrins [0–2]	1 [0–1]	1 [0–1]
Coproporphyrin I [0–15]	16 [9–17]	19 [17–22]
Coproporphyrin III [0–49]	8 [0–12]	36* [30–51]
Total coproporphyrins (I + III) [0–64]	18 [16–25]	56* [49–73]
Ratios:		
Heptacarboxyporphyrin/uroporphyrins	0.19 [0–0.25]	0.14 [0.09–0.25]
Hexacarboxyporphyrin/uroporphyrins	0 [0–0]	0 [0–0]
Pentacarboxyporphyrin/uroporphyrins	0.12 [0–0.2]	0.62 [0–0.11]
Coproporphyrin I/uroporphyrins	1.9 [1.1–2.2]	2.8* [2.3–4.5]
Coproporphyrin III/uroporphyrins	1.8 [0–6.3]	6.0* [4.6–11]
Total Coproporphyrins (I + III)/uroporphyrins	3.5 [1.4–8.3]	10* [7.2–13]

Note. IQR, interquartile range; NS, not significant. Asterisk indicates significantly different from mild ($p < .05$).
LabCorp testing revealed a significant correlation between LabCorp and the Laboratoire Philippe Auguste for the
ratios of total cP (I + III)/uP ($\tau_b = 0.31$, $p < .05$) and for the ratios of total cP (I + III)/uP (LabCorp) and prcP/uP
(Laboratoire Philippe Auguste) ($\tau_b = 0.31$, $p < .05$).

[a]Mild ASD is defined as any study participant with a CARS score less than the overall study participant median
(CARS score <39.25).

[b]Severe ASD is defined as any study participant with a CARS score greater than the overall study participant
median (CARS score >39.25).

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

significantly increased among participants with severe ASD in
comparison to participants with mild ASD. It was also
observed that there was a nonsignificant trend toward an
increasing median ratio of 5cxP/uP (5.2-fold) among partici-
pants with severe ASD in comparison to participants with mild
ASD. In contrast, no significant differences were observed for
any of the other urinary porphyrins measured among partici-
pants with severe ASD in comparison to mild ASD. It was also
observed when comparing urinary poprhyrins that were found
to be significantly elevated using LabCorp testing that there
was a significant correlation between LabCorp and the Labora-
toire Philippe Auguste for the ratios of total cP (I + III)/uP and
for the ratios of total cP (I + III)/uP (LabCorp) and prcP/uP
(Laboratoire Philippe Auguste).

Figure 1 shows the correlation between CARS scores and
urinary porphyrin levels among the participants examined. It
was found that there was a significant correlation were
between the CARS scores and the ratio of total cP (I + III)/uP.
In contrast, no significant correlation was observed between
the CARS scores and 7cxP/uP ratios.

DISCUSSION

The overall results of the present study demonstrated a sig-
nificant correlation between the clinical severity of ASD and
specific urinary porphyrins. It was observed that these effects
were significant when measuring the urinary porphyrin metab-
olites per liter of total urine volume or when evaluating their
ratio to uP levels (a measure of porphyrin synthesis rate). Fur-
ther, it was observed that there were roughly consistent mea-
surements for the urinary porphyrins performed both at
LabCorp and at a second laboratory, the Laboratoire Philippe
Auguste. Finally, for those urinary porphyrins that were found
to significantly correlate with the severity of ASD, an exami-
nation revealed a significant correlation between such porphy-
rins measured at LabCorp with prcP (a specific marker of Hg
body burden) measured at the Laboratoire Philippe Auguste.

Previous studies noted that distinct changes in urinary por-
phyrin concentrations were observed as early as 1–2 wk after
initiation of Hg exposure, and that these changes increased in a
dose- and time-related fashion with the concentration of Hg in
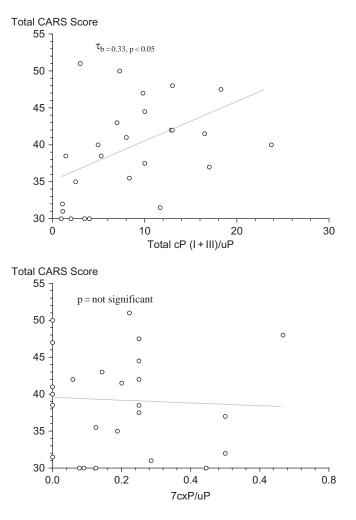the kidney, one of the principal target organs of Hg compounds

FIG. 1. A summary of the correlation between urinary porphyrin ratios and CARS scores.

gram creatinine in the urine, per liter urine, or per microgram uP in the urine).

The results of the present study are consistent with observations that Hg-associated urinary pophryin profiles significantly increase across the autism spectrum from individuals with a mild ASD diagnosis to those with a severe ASD diagnosis (Geier & Geier, 2006, 2007b; Nataf et al., 2006, 2008; Geier et al., 2009). Previous studies also demonstrated that chelation therapy in those with an ASD diagnosis resulted in significant reductions in these Hg-associated urinary porphyrin profiles (Geier & Geier, 2006, 2007b; Nataf et al., 2006). The results of the present study contextualize these previous findings by evaluating patients diagnosed with an ASD using CARS, a recognized test of ASD severity, prior to blinded laboratory testing; this study demonstrates a significant increasing correlation between Hg-associated urinary porphyrin profiles and ASD severity. In contrast, the urinary porhyrins that are not associated with Hg toxicity do not correlate with the child's autism severity score (see Figure 1). The present study demonstrated a correlation between a human clinical biomarker, previously shown to be associated with Hg body burden, and greater severity of ASD symptoms.

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 Hg body burden in individuals diagnosed with an ASD, are compatible with previous data showing, among individuals diagnosed with an ASD relative to controls: elevated brain Hg levels (Sajdel-Sulkowska et al., 2008); increased hair Hg levels (Fido & Al-Saad, 2005); elevated blood Hg levels (DeSoto & Hitlan, 2007); higher Hg levels in baby teeth (Adams et al., 2007); decreased excretion of Hg through first baby haircuts (Holmes et al., 2003; Adams et al., 2008); and increased Hg in the urine/fecal samples following chelation therapy (Bradstreet et al., 2003; Geier & Geier, 2007a). Furthermore, the approximately two- to threefold significantly elevated levels of Hg associated with urinary porphyrins are quantitatively compatible with the increased levels of Hg observed in the aforementioned studies.

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 laboratory 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

(Woods, 1996). 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 (Echeverria et al., 1995; Gonzalez-Ramirez et al., 1995; Woods, 1996; Pingree et al., 2001). It was previously concluded that urinary porphyrin profiles are a useful biomarker for Hg body burden and its potential adverse health effects in human subjects (Woods et al., 1993).

Several previous studies have examined urinary porphyrin profiles in individuals diagnosed with an ASD (Geier & Geier, 2006, 2007b; Nataf et al., 2006, 2008; Austin & Shandley, 2008; Geier et al., 2009). The results of this present study showing elevated urinary cP (I + III) levels are consistent with several of the aforementioned studies of individuals diagnosed with an ASD. Further, the present study also found, as was observed previously, that specific urinary porphyrin elevations were observed regardless of the urinary reference used (i.e., per

study were selected from the community of Dallas/Fort Worth. Third, and most importantly, the consistency and specificity of the results observed were strengths of the present study. This was especially shown with regards to the fact that the urinary porphyrin elevations observed were consistently elevated of similar magnitude regardless if they were measured per liter of urine or when compared in ratio to the quantity of uP present in the sample. Further, the directions of the significant effects observed were all in the biologically plausible direction, which is very unlikely to be a random occurrence. Finally, a further strength of the present study is that LabCorp and the Laboratoire Philippe Auguste are commercial laboratories that had to meet well-established standards of CLIA and ISO, respectively.

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 whether there were potential correlations between the urinary porphyrins examined and other potential biomarkers that indicate conditions such as oxidative stress or heavy metal toxicity among individuals diagnosed with an ASD. Further, while the present urinary porphyrin patterns observed are significantly suggestive of elevated Hg body burden among the individuals diagnosed with an ASD examined in the present study, and are consistent with previously cited studies showing elevated Hg body burden among individuals diagnosed with an ASD, it is possible that other environmental and/or genetic factors may be involved. Finally, another potential limitation of the present study was that for the overall urinary porphyrin values obtained for the ASD group examined, there was no normal control group to compare them against. Despite the lack of a normal control group, the present study did demonstrate that within the ASD group examined there was a significant correlation between ASD severity and urinary poprhyrins.

CONCLUSIONS

The present study was a prospective study conducted to evaluate urinary porphyrins in a cohort of patients diagnosed with an ASD, using routinely available clinical laboratory testing. For the study participants examined, this study found that increasingly severe ASD correlated with increasing levels of urinary porphyrins associated with Hg body burden. In addition, urinary porphyrin results among these same study participants, as measured by both LabCorp and the Laboratoire Philippe Auguste, were consistent. It is recommended that future studies need to focus on further evaluating urinary porphyrins metabolites in an expanded cohort of individuals

diagnosed with an ASD and that possible treatment protocols be evaluated for their potential to correct the urinary porphyrin abnormalities observed in the present study. Finally, since the laboratory testing employed in the present study for examining urinary porphyrins is clinically available (covered by many insurance companies in the United States), relatively inexpensive (under $200 per test), and relatively noninvasive, it is recommended that patients diagnosed with an ASD need to be routinely tested for urinary porphyrins to evaluate their present heavy metal body burden.

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