

A biomarker of mercury body-burden correlated with diagnostic domain specific clinical symptoms of autism spectrum disorder

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Abstract The study purpose was to compare the quantitative results from tests for urinary porphyrins, where some of these porphyrins are known biomarkers of heavy metal toxicity, to the independent assessments from a recognized quantitative measurement, the Autism Treatment Evaluation Checklist (ATEC), of specific domains of autistic disorders symptoms (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior) in a group of children having a clinical diagnosis of

autism spectrum disorder (ASD). After a Childhood Autism Rating Scale (CARS) evaluation to assess the development of each child in this study and aid in confirming their classification, and an ATEC was completed by a parent, a urinary porphyrin profile sample was collected and sent out for blinded analysis. Urinary porphyrins from twenty-four children, 2–13 years of age, diagnosed with autism or PDD-NOS were compared to their ATEC scores as well as their scores in the specific domains (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior) assessed by ATEC. Their urinary porphyrin samples were evaluated at Laboratoire Philippe Auguste (which is an ISO-approved clinical laboratory). The results of the study indicated that the participants' overall ATEC scores and their scores on each of the ATEC subscales (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior) were linearly related to urinary porphyrins associated with mercury toxicity. The results show an association between the apparent level of mercury toxicity as measured by recognized urinary porphyrin biomarkers of mercury toxicity and the magnitude of the specific hallmark features of autism as assessed by ATEC.

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Introduction

The American Psychiatric Association's Diagnostic Statistical Manual of Mental Disorders, 4th Edition Text-Revised (DSM-IV-TR) (American Psychiatric Association 2000) is the main diagnostic reference used by mental health professionals and insurance providers in the United States to diagnose patients with an autistic disorder. The diagnosis of an autistic disorder requires that at least six developmental and behavioral characteristics are apparent, that delays or abnormal functioning in at least one of the following areas (with onset prior to age 3 years) including: social interaction, language as used in social communication, or symbolic or imaginative play, and finally, that the disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder.

The following is from the DSM-IV-TR criteria for Autistic Disorder: "(1) qualitative impairment in social interaction, as manifested by at least two of the following: (a) marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction; (b) failure to develop peer relationships appropriate to development level; (c) a lack of spontaneous seeking to share enjoyment, interest, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest); (d) lack of social or emotional reciprocity. (2) qualitative impairments in communication as manifested by at least one of the following: (a) delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime); (b) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others; (c) stereotyped and repetitive use of language or idiosyncratic language (d) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level. (3) restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following: (a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus; (b) apparently inflexible adherence to specific, nonfunctional routines or rituals; (c) stereotypes and repetitive motor mannerisms (e.g., hand or finger flapping or twisting,

or complex whole-body movements); (d) persistent preoccupation with parts of objects."

As a result of the aforementioned criteria, it is imperative when evaluating any potential cause/contributing factors for autistic disorders that there be a significant correlation between the potential cause/contributing factor and the clinical symptoms that define autistic disorders. Previous studies have suggested a significant role for mercury (Hg) exposure as a cause/contributing factor for the development of autistic disorders (Bradstreet et al. 2003). DeSoto and Hitlan (2007), for example, found that children with ASD have higher levels of mercury in their blood than typically developing children and Adams et al. (2007) found that children with an ASD had higher levels of mercury in their baby teeth.

Mercury can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining/associated with autistic disorders, and these similarities extend to neuroanatomy, neurotransmitters, and biochemistry (Stringari et al. 2008; Toimela et al. 2004; Waly et al. 2004; Blaylock 2008; Blaylock and Struneka 2009; Danscher et al. 1990; Fonfria et al. 2005; Huang et al. 2008; Olanow and Arendash 1994). Furthermore, a review of molecular mechanisms indicates that Hg exposure can induce death, disorganization and/or damage to selected neurons in the brain similar to that seen in recent brain pathology studies of patients diagnosed with an ASD, and this alteration may likely produce the symptoms by which ASDs are diagnosed (Lopez-Hertado and Prieto 2008; Kern 2003; Kern and Jones 2006).

The purpose of the present study was to utilize a known biomarker of heavy metal body-burden in comparison to a recognized quantitative measurement of specific domains of ASD symptoms (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior).

Materials and methods

Overview

The study was conducted at the Autism Treatment Center (Dallas, TX). The study protocol received Institutional Review Board (IRB) approval from the Liberty IRB, Inc. (Deland, FL). All parents of

subjects examined in the present study 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.

After a confirmatory CARS (Schopler et al. 1994) evaluation was conducted on each participant in the study by the principal investigator (JKK) and a parent completed an ATEC (Rimland and Edelson 1999), a first-morning-urine sample was collected and submitted to Laboratoire Philippe Auguste for a standard urinary porphyrin profile assessment. Then, the urine porphyrin results reported by this laboratory were tabulated and compared to the scores from the ATEC.

Participants

The present study looked at consecutive qualifying participants ($n = 24$) who were prospectively recruited from the community of Dallas/Fort Worth. All of the children had a diagnosis of autism or pervasive developmental disorder (PDD) and were not previously chelated. Children included in the present study were between 2 and 13 years of age and had an initial CARS (Schopler et al. 1994) score of ≥ 30 (where a score of 30 is defined as the lower bound for an ASD diagnosis) as determined by Dr. Kern at baseline (initial intake) based upon observation of the study subjects and interviewing the parent(s). Dr. Kern has been formally trained in the use of CARS and has 12 years of experience in using CARS to evaluate hundreds of children diagnosed with an ASD. Though none were encountered, this study was designed to exclude children who had 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

Among qualifying subjects, the subject's parent completed an ATEC (Rimland and Edelson 1999) form developed by the Autism Research Institute (San Diego, CA). This measure was completed by a parent about a day or two before the collection of

the urine. The ATEC consists of four subtest scales: Scale I. Speech/Language/Communication (14 items—scores can range from 0 to 28), Scale II. Sociability (20 items—scores can range from 0 to 40), Scale III. Sensory/Cognitive Awareness (18 items—scores can range from 0 to 36), and Scale IV. Health/Physical/Behavior (25 items—scores can range from 0 to 75). The four subscale scores can be used to calculate a total score (total scores can range from 0 to 180). The scores are weighted according to the response and the corresponding subscale. The higher the subscale and total score, the more impaired the subject. The lower the subscale and total score, the less impaired the subject. The overall scores in each subscale and the total score can be extrapolated to determine the percentile of severity of the subject in comparison to score distributions provided by the Autism Research Institute. Finally, Pearson split-half (internal consistency) coefficients provided by the Autism Research Institute based upon evaluation of 1,358 subjects revealed uncorrected r values as follows: Scale I. Speech/Language/Communication (0.920), Scale II. Sociability (0.836), Scale III. Sensory/Cognitive Awareness (0.875), Scale IV. Health/Physical/Behavior (0.815), and total score (0.942).

Lab evaluation

Following the intake CARS and ATEC evaluations, a urine sample was collected from each subject. The laboratory specimens were all collected in the morning (as first morning urine samples) following an overnight fast. Specimens were shipped to the Laboratoire Philippe Auguste (Paris, France). The lab used in the present study was blinded and received no information regarding the clinical status of the subjects examined or their ATEC or CARS scores.

Participants were tested for the following at Laboratoire Philippe Auguste, Paris, France (ISO-approved) urinary porphyrin testing including—uroporphyrin (uP), heptacarboxyporphyrin (7cP), hexacarboxyporphyrin (6cP), pentacarboxyporphyrin (5cP), precoproporphyrin (PrcP), and coproporphyrin (cP). Each specific type of porphyrin examined in the present study was evaluated as a ratio to uP levels (nmol porphyrin:nmol porphyrin). In addition, the ratio of $(5cP + PrcP)/(7cP + uP)$ was also examined

because it is an overall biomarker associated with mercury toxicity.

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 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 HPLC Spectrofluorimetric technique: after centrifugation ($3000 \times g$, 5 min), 500 μl urine were 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 KH_2PO_4 , pH adjusted to 3.5 with CH_3COOH ; Phase B: CH_3OH). Eluant flow was 1 ml/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 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, prcP, and cP, respectively (Fig. 1). The HPLC separation method used by this lab 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, Utah). The urinary creatinine level in each sample was measured by a spectrophotometric assay (Vitros, Ortho-Clinical Diagnostics, Johnson and Johnson). The porphyrin values reported were expressed in nanomoles per g of creatinine measured (Nataf et al. 2006).

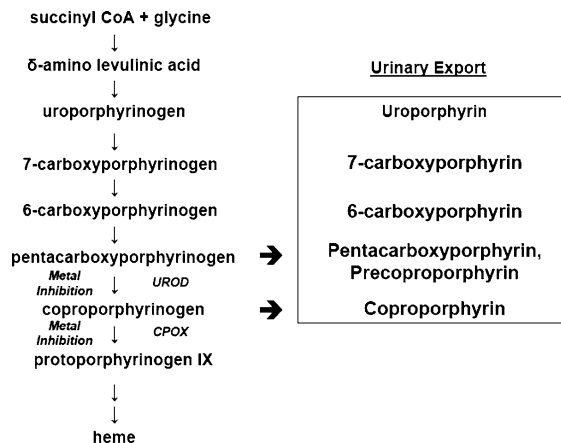


Fig. 1 A summary of the heme synthesis pathway and major urinary metabolites (Nataf et al. 2006). Porphyrinogens appear in urine as porphyrin derivatives (right). Mercury can cause increased urinary 5cxP, prcP, and cP by inhibiting uroporphyrinogen decarboxylase (UROD) and/or coproporphyrinogen oxidase (CPOX); urinary uroporphyrin is not reported to alter with inhibition of these enzymatic steps

Statistical analyses

The statistical package SAS (version 9.1) was utilized. In all statistical analyses a two-tailed P -value of ≤ 0.05 was considered statistically significant. In the present study, linear regression was used to assess the correlation between each of the urinary porphyrin measures and each of the five different ATEC-subscale scores. In each instance age, gender, race (white/nonwhite), and supplements (taking/not taking) were included as covariates. Race was collapsed into two categories because there were few non-white subjects and year of birth was not included because it was highly correlated with age. Residuals and graphs from each regression analysis were checked for outliers. One subject had a very high value for 5cP (13.02) which had a large effect on the slope for this predictor. Based on the abnormally high magnitude of the 5cP value for this subject, this datapoint was deleted from the data for all the regression instances in which it was a variable or part of a variable.

Results

Table 1 presents the demographic characteristics of the sample. Table 2 evaluates the relationships between

Table 1 A summary of the subjects diagnosed with an autistic disorder

Descriptive information	Overall (<i>n</i> = 24)
Sex/age	
Male/female (ratio)	20/4 (5:1)
Mean age in years \pm Std (range)	5.75 \pm 2.79 (2–13)
Race (<i>n</i>)	
Caucasian	67% (16)
Minorities ^a	33% (8)
Previous treatments (<i>n</i>)	
Supplements	33% (8)
Autism Treatment Evaluation Checklist Scores^b	
Overall	56.75 \pm 22.30 (30–39th) ^c
Speech/language/communication	10.58 \pm 6.93 (40–49th)
Sociability	13.08 \pm 6.50 (40–49th)
Sensory/cognitive awareness	14.21 \pm 6.71 (40–49th)
Health/physical/behavior	18.88 \pm 7.71 (40–49th)
Urinary porphyrins (nmol/nmol)^d	
7cP/uP	0.20 \pm 0.07
6cP/uP	0.05 \pm 0.02
5cP/uP	0.23 \pm 0.08
PrcP/uP	0.93 \pm 0.34
cP/uP	11.31 \pm 3.74
(5cP + PrcP)/(7cP + uP)	0.95 \pm 0.31

Std = standard deviation

All participants examined in the present study were living in the state of Texas and had not previously received chelation therapy

^a Includes participants of Hispanic, Black, Asian, or Mixed Ancestry

^b Mean \pm standard deviation. Study subject parents completed the Autism Treatment Evaluation Checklist prior to lab testing

^c Percentile of Severity (the higher the number, the more severe the clinical symptoms)

^d Mean \pm standard deviation. Urinary porphyrins were measured by the Laboratoire Philippe Auguste (Paris, France) blinded as to the diagnosis/clinical severity of the subjects

the different urinary porphyrins measurements to predict each of the five different ATEC scores examined in the present study. Table 3 is a summary of adjusted correlations observed between urinary porphyrins and autism symptom scores. The urinary porphyrin parameters that were found to be significantly associated with the overall ATEC scores were 5cP/uP, PrcP/uP, and the ratio (5cP + PrcP)/(7cP + uP). The urinary porphyrin parameters that were significantly associated with ATEC Speech/

Language subscale were PrcP/uP and the ratio (5cP + PrcP)/(7cP + uP). The urinary porphyrin parameters that were significantly associated with ATEC Sociability subscale were PrcP/uP and the ratio (5cP + PrcP)/(7cP + uP). The urinary porphyrin parameters that were significantly associated with ATEC Sensory/Cognitive Awareness subscale were PrcP/uP and the ratio (5cP + PrcP)/(7cP + uP). The urinary porphyrin parameters that were significantly associated with ATEC Health/Physical/Behavior subscale were 7cP/uP and 5cP/uP.

Discussion

The results of the study showed that the participants' overall ATEC scores and their scores on each of the ATEC subscales (Speech/Language, Sociability, Sensory/Cognitive Awareness, and Health/Physical/Behavior) had a linear relationship with some ratio measures of the mercury-associated porphyrin(s) (5cP, PrcP and [5cP + PrcP]) to some non-mercury-associated porphyrin(s) (uP and [7cP + uP]). The ATEC Health/Physical/Behavior subscale also showed a relationship with 7cP/uP. This urinary porphyrin (7cP/uP) is considered to be indicative of arsenic and certain organic chemical (such as polychlorinated Biphenol (PCB)). No other non-mercury-associated porphyrin, i.e., uP or 6cP, showed an association with the ATEC overall or any of the subscales. The results of the present study suggest that mercury-associated porphyrins are associated with the features of autism.

The ATEC examines the four domains that are affected in autism and provides a score as to the level of difficulty the child has in that area. For example, the Speech and Language score quantitatively shows how severely the child is affected in the use of language and communication, e.g., does the child have speech, can the child use words meaningfully, can the child use the words to communicate with others? The Sociability domain quantitatively describes the child's ability to interact with others. The Sensory domain shows the extent of difficulty the child has with processing sensory information and understanding their world. The Health/Physical/Behavioral section quantitatively describes the daily problems that the child and their families have to confront such as incontinence, inability to sleep, screaming, agitation, etc. Although, the ATEC is not a direct measure of

Table 2 Urinary porphyrins as a predictor of Autism Treatment Evaluation Checklist scores

Urinary porphyrins	Overall	Speech/language/communication	Sociability	Sensory/cognitive awareness	Health/physical/behavior
7cP/uP					
Slope	1.03 ^{###}	0.16 ^{###}	0.17 ^{###}	0.19 ^{###}	0.51
Standard error	0.6	0.2	0.2	0.2	0.2
<i>T</i> -Statistic	1.8	0.9	0.9	1.0	2.6
<i>P</i> -Value	0.09	0.41	0.38	0.35	0.02
6cP/uP					
Slope	0.05 ^{###}	0.19 ^{###}	-0.34 ^{###}	-0.09 ^{###}	0.28
Standard error	2.2	0.7	0.7	0.7	0.8
<i>T</i> -Statistic	0.0	0.3	-0.5	-0.1	0.3
<i>P</i> -Value	0.98	0.79	0.63	0.91	0.73
5cP/uP					
Slope	1.05^{###}	0.10 ^{###}	0.27 ^{###}	0.19 ^{###}	0.49
Standard error	0.5	0.2	0.2	0.2	0.2
<i>T</i> -Statistic	2.3	0.6	1.8	1.1	3.2
<i>P</i> -Value	0.04	0.56	0.09	0.28	0.005
PrcP/uP					
Slope	3.46^{###}	0.96^{##}	0.88^{##}	1.00^{###}	0.63 ^{##}
Standard error	1.2	0.4	0.4	0.4	0.5
<i>T</i> -Statistic	3.0	2.5	2.2	2.5	1.3
<i>P</i> -Value	0.01	0.02	0.04	0.02	0.22
cP/uP					
Slope	2.16	0.52	0.54	0.52	0.58
Standard error	1.2	0.4	0.4	0.4	0.4
<i>T</i> -Statistic	1.8	1.3	1.4	1.3	1.3
<i>P</i> -Value	0.08	0.20	0.18	0.22	0.21
(PrcP + 5cP)/(7cP + uP)					
Slope	3.65^{###}	0.94^{##}	0.99^{##}	1.04^{##}	0.67 ^{##}
Standard error	1.3	0.4	0.4	0.5	0.6
<i>T</i> -Statistic	2.7	2.1	2.2	2.3	1.2
<i>P</i> -Value	0.01	0.049	0.04	0.04	0.24

ATEC = Autism Treatment Evaluation Checklist; 7cP = heptacarboxyporphyrin; 6cP = hexacarboxyporphyrin; 5cP = pentacarboxyporphyrin; PrcP = precoproporphyrin; cP = coproporphyrin; uP = uroporphyrin

The linear regression statistic was utilized to construct models with adjustments for age, gender, race, and supplementation status

[#] Slope represents change in ATEC score per 10-point change in urinary porphyrin measure

^{##} Slope represents change in ATEC score per 0.1 point change in urinary porphyrin measure

^{###} Slope represents change in ATEC score per 0.01 point change in urinary porphyrin measure

brain dysfunction, the ATEC can indirectly show that the child's brain has limitations in these specific areas and the extent of the impairment.

By the same token, urinary porphyrins do not directly measure or exactly identify the environmental toxins. Instead, urinary porphyrin patterns identify

profiles well known to be associated with certain types of environmental toxins, including mercury, and subsequently can be used to quantify a level of toxicity. For example, Woods (1996) noted that urinary mercury-associated porphyrin concentrations increased in a dose- and time-related fashion with the

Table 3 A summary of adjusted correlations observed between urinary porphyrins and autism symptom scores

ATEC domains	7cP/uP	6cP/uP	5cP/uP	PrcP/uP	cP/uP	(PrcP + 5cP)/(7cP + uP)
Overall			*	**		*
Speech/language/communication				*		*
Sociability				*		*
Sensory/cognitive awareness				*		*
Health/physical/behavior	*		**			

ATEC = Autism Treatment Evaluation Checklist; 7cP = heptacarboxyporphyrin; 6cP = hexacarboxyporphyrin; 5cP = pentacarboxyporphyrin; PrcP = precoproporphyrin; cP = coproporphyrin; uP = uroporphyrin

The linear regression statistic was utilized to construct models with adjustments for age, gender, race, and supplementation status

* $P < 0.05$

** $P < 0.01$

concentration of mercury in the kidney, a principal target organ of mercury compounds.

Although this is not a direct measure of mercury in the brain, studies show that the brain and kidneys are target organs for mercury following mercury exposure. For example, Pingree et al. (2001) gave rats methylmercury hydroxide (MMH) (10 ppm) in drinking water for 9 weeks and determined both inorganic (Hg^{2+}) and organic (CH_3Hg^+) mercury 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.

Furthermore, previous studies have reported on the utility of urinary porphyrins to assess the relationship between neurobehavioral problems and mercury body-burden (Woods 1996). Echeverria et al. (1995) examined the behavioral effects of low-level exposure to mercury vapor among dentists. These investigators observed that urinary porphyrins were as sensitive as urinary mercury levels for observing adverse effects of mercury on cognitive and motor testing. In addition, Woods et al. (2009) recently published a study supporting the sensitivity of urinary porphyrins as a biological indicator of subclinical mercury exposure in children.

The results of the current study that show a correlation between the severity of each of the ATEC domains and the level of toxicity suggests that ASD has an environmental component. Although the influence of genetics or predisposing vulnerability factors cannot be ruled out, the results of this study suggest that toxicity plays a role and that ASD may be a result of neuronal insult.

Strengths and limitations

The present study does have the limitation that it was not possible to directly measure or exactly identify the source of the environmental toxins examined. Instead, urinary porphyrin patterns were examined to identify profiles well known to be associated with certain types of environmental toxins, including mercury. As a result, it is possible that there may be certain genetic or other environmental contributing factors to the effects observed. In addition, another limitation of the present study is that there may be confounding variables present in the data that were not identified. A significant effort was made to adjust for standard potential confounders in the present dataset, because each correlation examined was adjusted for the gender, age, race, and supplementation status of the subject.

The central strength of the present study stems from its design as a prospective, blinded study. As a result, it was not possible for investigators to directly or indirectly influence the collection of study participants examined. In addition, since two-tailed statistical testing was used, a P -value ≤ 0.05 was considered statistically significant, and the overall sample size was of moderate size, it is unlikely that the effects observed were due to mere statistical chance.

Conclusion

Mercury is a neurotoxin that has been used in agriculture, as a preservative in vaccines, and medicinally and released into the environment in the burning

of coal and the production of chlorine. There has been a dramatic increase in exposure to mercury in the last twenty years (Schuster et al. 2002), an increase in detected blood mercury levels in the US population (Laks 2009), and a dramatic increase in the rates of autism (Kalia 2008; Hertz-Picciotto and Delwiche 2009; Hertz-Picciotto 2009; Rutter 2005). Many studies have shown an association between mercury and autism (Lopez-Hertado and Prieto 2008; DeSoto and Hitlan 2007; Adams et al. 2007), and some studies have also indicated a role of other toxic metals (Adams et al. 2009); however, this present study takes the investigation one step further and shows an association between mercury toxicity and the specific hallmark features of autism and PDD-NOS.

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Conflict of interest statement David Geier, Janet Kern, and Mark Geier have been involved in vaccine/biologic litigation.

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