INTRODUCTION

Autism spectrum disorders (ASDs) are neurodevelopmental disorders, presenting in childhood that affect at least 1 in 110 children in the United States (Centers for Disease Control and Prevention 2009). The condition is characterized by severe impairments in socialization, communication, and behavior. Individuals diagnosed with an ASD may display a range of problem behaviors such as hyperactivity, poor attention, impulsivity, aggression, self-injury, and tantrums. Further, these children often display unusual responses to sensory stimuli, such as hypersensitivities to light, sound, color, smell or touch, and have a high threshold to pain (Austin 2008).

Emerging evidence supports the theory that some ASDs may result from a combination of genetic/biochemical susceptibility, specifically a reduced ability to excrete mercury (Hg), and exposure to Hg at critical developmental periods (Austin 2008, Geier et al. 2008, 2009e). Exposure to Hg can cause immune, sensory, neurological, motor, and behavioral dysfunctions similar to traits defining/associated with ASDs, and these similarities extend to neuroanatomy, neurotransmitters, and biochemistry (Austin 2008, Geier et al. 2008, 2009e).

DeSoto and Hitlan (2007) postulated that if Hg does play any causal role in facilitating an ASD diagnosis, there would likely be at least some correlation between high Hg measured in the blood and the symptoms of autism, even if an individual’s ability to metabolize mercury mediates the relationship between exposure and neural toxicity. This is because even if exposure is identical, those who remove Hg less effectively should still have higher levels in the blood. Subsequently, these researchers analyzed blood Hg levels in a cohort of children from China (ASDs and controls). These researchers concluded that a statistically significant relationship exists between total blood Hg levels and a diagnosis of an ASD (DeSoto and Hitlan 2007).

A subsequent study by Hertz-Picciotto and coauthors (2010) examined blood Hg levels documented in
lab results from the Childhood Autism Risk from Genetics and the Environment (CHARGE) study. These investigators examined three groups of children \((n=452)\): ASD, non-ASD with developmental delay (DD), and population-based neurotypical controls. Mothers were interviewed about household, medical and dietary exposures. The geometric mean for typically developing children \(0.28 \mu g/L\), was significantly higher than for ASD \(0.19 \mu g/L, P=0.006\) or DD \(0.17 \mu g/L, P=0.01\) children; after adjustment for demographic factors and Hg sources and application of weights, the geometric means for ASD, DD and neurotypical children were 0.26, 0.16, and 0.24 \mu g/L, respectively, with only the DD group significantly different from controls \(P=0.007\).

The purpose of the present study was to evaluate the consistency of blood Hg measurements made by a clinical lab in the US with those previously observed among Chinese children by DeSoto and Hitlan (2007) and US children, by Hertz-Picciotto and colleagues (2010), by examining US children (ASDs and controls) for their blood Hg levels. Further, the present study also evaluated blood Hg measurements made by a clinical lab in the US to determine if there was a threshold blood Hg level that significantly increased the risk of an ASD diagnosis.

**METHODS**

**Study participants**

In the present study, a retrospective examination of lab result reports generated from blood red blood cell (RBC) total Hg levels, measured by Vitamin Diagnostics (Cliffwood Beach, NJ, CLIA-approved), was undertaken. The lab result reports were generated from 2003 through 2007. The examination in the present study was confined to lab reports with a physician-diagnosed International Classification of Disease, 9th revision (ICD-9) ASD (299.00, 299.80) and reports from neurotypical control children. Blood samples taken from children diagnosed with an ASD were submitted by the child’s physician and/or healthcare provider for analysis as part of the child’s routine medical care. Blood samples for neurotypical control children were collected from patients at ‘well’ medical check-ups, after appropriate consent was obtained, in order to help Vitamin Diagnostics establish normal reference ranges for lab tests. In addition, some blood samples for neurotypical control children were submitted by referring physicians who routinely utilized Vitamin Diagnostics lab testing. A total cohort of 172 children was examined in the present study. Information gleaned from the lab reports included blood RBC total Hg levels, age, sex, and sample collection date. Table I summarizes the demographic information regarding the patients examined in the present study.

### Table I

<table>
<thead>
<tr>
<th>Diagnosis (ICD-9 Code)</th>
<th>(n)</th>
<th>Mean Age ± SD (Range)</th>
<th>Percent Male</th>
<th>Mean Year Sample Collected ± SD (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autism Spectrum Disorder (299.00, 299.80)</td>
<td>83</td>
<td>7.3 ± 3.7 (2.3–15.2)</td>
<td>69.9%</td>
<td>2005 ± 1.4 (2003–2007)</td>
</tr>
<tr>
<td>Neurotypical Control</td>
<td>89</td>
<td>11.4 ± 2.2 (6.5–15.3)</td>
<td>78.7%</td>
<td>2004 ± 1.2 (2003–2007)</td>
</tr>
</tbody>
</table>

(SD) standard deviation

**Lab evaluation**

In order to examine blood RBC total Hg levels, venous blood from the antecubital veins of subjects were collected in a royal blue top tube with EDTA (BD Trace Metal Tube #1125001), using a 22-gauge needle. Approximately 20 minutes after the blood draw, the tubes were centrifuged at 1 800 rpm for at least 20 minutes. The stoppers of the tubes were removed and the plasma and the white cell layers were discarded. The
packed red cells were shipped overnight from the doctor’s office. Immediately upon arrival, the RBC was treated with equal volume of HEPES-Na-buffered solution (HBS; 133.5 mM NaCl, 2 mM glucose and 15 mM HEPES-Na buffer, pH 7.4) supplemented with 1 mM EDTA and 0.1% bovine serum albumin (BSA).

After centrifugation at 1 000 × g for 10 min at 25°C, the packed RBCs were resuspended with HBS supplemented with 0.01% BSA (HBS-BSA) and subsequently washed two times in HBS-BSA by repeated resuspension and centrifugation at 800 × g for 10 minutes at 25°C. Finally, the RBC suspension was prepared with HBS-BSA, the cell density of which was adjusted to 8×10⁹ cells/mL and stored at 22°C. All experiments were performed within 48 hours of the collection of the blood samples.

RBCs were lysed using Lysing buffer of BD science (#555899). The samples were analyzed using a flow injection cold vapor Hg analysis system (Perkin-Elmer Corporation, Shelton, CT) that consisted of the following components: a model 3100 spectrophotometer equipped with a 19 cm flow cell maintained at 100°C, an FIAS 200 pump system with a 1 mL sample loop, an AS-91 Auto Sampler, and an EDL system 2 lamp power supply. The system was controlled with a Digital Equipment DEC station 325C computer and Perkin-Elmer Controlling software. The total Hg analyses used sodium borohydride as the reducing agent. A detailed description has been published previously (Chen et al. 1998).

In addition, to evaluate the accuracy of blood RBC total Hg levels measured at Vitamin Diagnostics, split blood samples for three specimens were sent to the University of Rochester (Rochester, NY) for analysis. Table II summarizes the comparative blood RBC total Hg levels determined by Vitamin Diagnostics and the University of Rochester. The results reveal order-of-magnitude comparable measurements between the two labs utilized for measuring blood RBC total Hg levels.

### Statistical analyses

The statistical package in SAS (version 9.1) was utilized. In all statistical analyses, a two-tailed $P$-value ≤0.05 was considered statistically significant. Statistical models were constructed in the present study to evaluate blood RBC total Hg levels among subjects diagnosed with an ASD in comparison to controls. The null hypothesis was that blood RBC total Hg levels would be similar among subjects diagnosed with an ASD and controls. The analysis of covariance test statistic was utilized to evaluate the mean blood RBC total Hg levels among subjects diagnosed with an ASD in comparison to controls, and the data were adjusted for age, gender, and sample collection date.

In addition, the relationship between blood RBC total Hg level and the risk of an ASD diagnosis was evaluated to determine if there was a threshold Hg level above which an increased risk of this disorder, relative to the controls examined, existed. The null hypotheses was that there would be no threshold blood RBC total Hg level above which there was an increased risk of this disorder relative to the controls examined. Logistic regression analysis was used because this statistical analysis approach is appropriate for the type of case/control study undertaken in the present study (Hosmer and Lemeshow 2000). To assess the form of the relationship, the data were divided into quintiles of Hg level and the odds of an ASD diagnosis, relative to the lowest level, were computed after adjustment for age, gender, and the year the data was collected. Since none of the controls had Hg levels at the highest quintile, the data values for the top two quintiles were combined.

### Table II

A summary of blood RBC total mercury analysis (split sample) in two different labs using a similar method of analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vitamin Diagnostics</th>
<th>University of Rochester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample – A</td>
<td>3.8 µg/L</td>
<td>3.1 µg/L</td>
</tr>
<tr>
<td>Sample – B</td>
<td>1.6 µg/L</td>
<td>1.2 µg/L</td>
</tr>
<tr>
<td>Sample – C</td>
<td>4.1 µg/L</td>
<td>3.8 µg/L</td>
</tr>
</tbody>
</table>
RESULTS

As summarized in Table I, the data used for this analysis consisted of those subjects diagnosed with an ASD (n=83) in addition to the controls (n=89). Males comprised 74.4% of the overall cohort examined (69.9% of cases and 78.6% of controls). The ASD subjects and the controls did not significantly differ by gender ($\chi^2 = 1.7$, df =1, $P = 0.19$). The overall mean age for the study participants was 9.4 ± 3.6 years (7.3 ± 3.7 years for the cases, 11.4 ± 2.2 years for the controls). The mean age difference between the two groups was statistically significant ($t=8.3$, df=170, $P < 0.0001$).

Table III summarizes the relative levels of RBC total Hg present among subjects diagnosed with an ASD in comparison to controls. The overall mean blood RBC total Hg level was 16.2 ± 10.6 µg/L for all participants (case and control). For subjects diagnosed with an ASD the mean blood RBC total Hg level was 22.2 ± 12.1 µg/L, while for controls, the mean blood RBC total Hg level was 10.7 ± 4.3 µg/L. After adjusting for age, gender, and year of sample collection, the mean blood RBC total Hg level was 21.4 µg/L for subjects diagnosed with an ASD and 11.4 µg/L for controls. The difference was statistically significant ($F=30.7$, df =1,171, $P < 0.0001$).

Table IV summarizes this relationship between increasing blood RBC total Hg levels and the risk of an ASD diagnosis in comparison to controls. The odds of ASD diagnosis were significantly greater than 1 only for the highest 2 quintiles (>15 µg/L). This result suggests that, on average, blood RBC total Hg level does not increase the odds of an ASD diagnosis until it is >15 µg/L. Fitting the model with a binary Hg level (above/below 15 µg/L), the odds of an ASD diagnosis for subjects with a blood RBC total Hg level >15 µg/L were 6.4 times greater than for subjects with a blood RBC total Hg level <15 µg/L ($\chi^2=14.3$, df=1, $P=0.0002$).

DISCUSSION

The results of the present study found that, on average, the subjects diagnosed with an ASD had significantly increased mean blood RBC total Hg levels in comparison to controls, even after adjusting for potential confounders such as age, gender, and date of sample collection. The present study also revealed that there was an apparent threshold effect of blood Hg levels for the risk of a study participant being diagnosed with an ASD. Overall, the present results are consistent with those measured by DeSoto and Hitlan (2007) but not those of Hertz-Picciotto and coauthors (2010).

In further considering the consistency of the results observed in the present study with those previously reported by DeSoto and Hitlan (2007), an analysis of the raw data from their study (Brumback 2007) was undertaken to evaluate if the current analysis methods employed would yield results similar to those observed in the present study. Consistent with the results from
the present study, after adjusting for age and gender, there was a significantly \( (F=4.8, \text{df}=1.136, P=0.030) \) higher mean total blood Hg level among autism cases (22.4 nmol/L) in comparison to controls (16.9 nmol/L) from the data used by DeSoto and Hitlan (2007). Further, the data used by DeSoto and Hitlan (2007) were divided into quintiles of total blood Hg level, and the odds of autism relative to the lowest level were computed using a logistic regression model which also contained terms for age and gender. Table V summarizes the results of the analysis. The odds of autism were only significantly greater than 1 for the highest quintile (>26 nmol/L). This result suggests that total blood Hg level does not increase the odds of autism until it is >26 nmol/L (>5.2 µg/L). This new model was fit with a binary Hg level that is 0 for levels <26 nmol/L and 1 for levels >26 nmol/L. The odds of autism for subjects with a total blood Hg level >26 nmol/L was 3.2 times greater than for subjects with a total blood Hg level <26 nmol/L \( (\chi^2=5.3, \text{df}=1, P=0.021) \).

In considering the apparent contradictory results obtained in the present study with those previously reported by Hertz-Picciotto and colleagues (2010), it is apparent that the Hertz-Picciotto and others (2010) study may have some significant limitations. Namely, Hertz-Picciotto and coauthors (2010) observed, in a non-biologically plausible finding, that without adjustment, children diagnosed with an ASD had a significant reduction in their blood Hg levels in comparison to neurotypical controls (32% reduction in blood Hg levels), and this same effect was apparent for children diagnosed with DD (39% reduction in blood Hg levels). Hertz-Picciotto and others (2010) recognized potential problems in their dataset, and subsequently, attempted to use specialized statistical methods to parse out behaviors associated with an ASD diagnosis that might have an effect on blood Hg levels. After the adjustments were made by Hertz-Picciotto and coworkers (2010), a non-significant increase in blood Hg levels among children diagnosed with an ASD in comparison to controls (8% increase in blood Hg levels) was observed, but children diagnosed with DD still had a significant reduction in their blood Hg levels in comparison to controls (33% reduction in blood Hg levels). Considering the consistent finding of non-biological plausible results by Hertz-Picciotto and colleagues (2010), even after significant adjustments, it is hard to interpret these investigators’ findings.

In further considering the consistency of findings made in the present study with previous studies, the present results are similar to observations made in a number of other studies on individuals diagnosed with an ASD. Specifically, the elevated blood Hg levels observed in the present study are compatible with previous data showing: increased brain Hg levels (Sajdel-Sulkowska et al. 2008); increased Hg levels in baby teeth (Adams et al. 2007); increased hair Hg levels (Fido and Al-Saad 2005); decreased excretion of Hg through first baby haircuts (Holmes et al. 2003, Adams et al. 2008); increased Hg in the urine/fecal samples following chelation therapy (Bradstreet et al. 2003, Geier and Geier 2007a); and increased Hg-associated

<table>
<thead>
<tr>
<th>Blood RBC Total Mercury Level (µg/L)</th>
<th>Adjusted(^1) Odds of an ASD Diagnosis</th>
<th>Adjusted(^1) P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–8.5</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>8.5–12.3</td>
<td>1.6</td>
<td>0.54</td>
</tr>
<tr>
<td>12.3–15</td>
<td>0.6</td>
<td>0.50</td>
</tr>
<tr>
<td>&gt;15</td>
<td>6.3</td>
<td>0.0067</td>
</tr>
</tbody>
</table>

Logistic regression analysis was used to evaluate the relationship between blood RBC mercury levels and the risk of an ASD diagnosis among the subjects examined. (RBC) red blood cell. \(^1\)Adjusted for the age, gender, and year of sample collection.
urinary porphyrins (Geier and Geier 2006, 2007b, Nataf et al. 2006, 2008, Austin and Shandley 2008, Geier et al. 2009b,c), among individuals diagnosed with an ASD relative to controls. Further, the about 2-fold significantly increased levels of blood Hg observed among subjects diagnosed with an ASD in comparison controls in the present study is quantitatively compatible with the increased levels of Hg observed in the aforementioned studies.

Furthermore, it was observed in blinded studies of children diagnosed with an ASD that the greater the Hg body burden (as measured by Hg-associated porphyrins), the more severely affected the child diagnosed with an ASD, as measured by a professional evaluation based upon the Childhood Autism Rating Scale (CARS) (Geier et al. 2009b,c). It was also observed from regression analyses that the body burden of toxic metals, particularly Hg, as assessed by urinary excretion before and after detoxification therapy, was significantly related autism severity, as measured by a professional evaluation based on the Autism Diagnostic Observation Schedule (ADOS), among children diagnosed with an ASD (Adams et al. 2009).

Many other studies, particularly in recent years, showed a significant relationship between Hg exposure and the risk of a child being diagnosed with an ASD (Austin 2008, Geier et al. 2008, 2009d). In the state of Texas, for example, a significant correlation was found between environmentally released Hg and the rate of ASD diagnoses by county (Palmer et al. 2006). A later study showed that the risk of a child being diagnosed with an ASD in Texas significantly correlated with the distance to a point source of Hg release from a power plant or a factory (Palmer et al. 2009). Other investigators have shown association between environmental Hg levels and the risk of an ASD diagnosis in the San Francisco Bay area (Windham et al. 2006). Finally, Schweikert and coauthors (2009) undertook an evaluation in the US, on a state by state basis, of ASD prevalence among 3 to 5 year-old children from 2000 to 2006 and environmental Hg exposure levels from 1996 to 2006. These investigators observed that Hg concentration in the environment among children 1 year-old or younger had a significant association with ASD prevalence three years later.

A review of the literature indicates that it is biologically plausible for increased Hg exposure to induce neurological damage similar to that observed in recent brain pathology studies of subjects diagnosed with an ASD (Kern and Jones 2006, Austin 2008, Evans et al. 2008, Geier et al. 2008, 2009a,e, Lopez-Hurtado and Prieto 2008, Sajdel-Sulkowska et al. 2008). Examples of the types of neurological insult resulting from Hg exposure, consistent with the neurological damage observed in brain pathology studies on ASD patients, include: (1) increased neuronal degeneration; (2) increased neuronal lipid peroxidation/oxidative stress; (3) increased neuronal excitotoxicity and cell death; (4) decreased neuronal growth factor signaling; (5) disruption in the neuronal antioxidant system; (6) reduced

<table>
<thead>
<tr>
<th>Total Blood Mercury Level (nmol/L)</th>
<th>Adjusted^1 Odds of an ASD Diagnosis</th>
<th>Adjusted^1 P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>6–9.8</td>
<td>0.8</td>
<td>0.78</td>
</tr>
<tr>
<td>9.8–18</td>
<td>1.0</td>
<td>0.95</td>
</tr>
<tr>
<td>18–26</td>
<td>1.5</td>
<td>0.46</td>
</tr>
<tr>
<td>&gt;26</td>
<td>3.4</td>
<td>0.0492</td>
</tr>
</tbody>
</table>

Logistic regression analysis was used to evaluate the relationship between total blood mercury levels and the risk of an ASD diagnosis among the subjects examined. ^1Adjusted for the age and gender.
neuronal glutathione and acetyl cholinesterase activities; and (7) neuronal necrosis, axonal demyelination, and gliosis. Further, recent animal studies have shown that low-dose Hg exposure induced brain pathology and clinical symptoms similar to those observed in subjects diagnosed with an ASD (Hornig et al. 2004, Burke et al. 2006, Falluel-Morel et al. 2007, Laurente et al. 2007, Bourdineaud et al. 2008, Fischer et al. 2008, Montgomery et al. 2008, Olczak et al. 2009).

Strengths/Limitations

The main strength of the present study is the consistency between blood mercury levels observed among the subjects examined in the US subjects with those observed previously on subjects examined in China (DeSoto and Hitlan 2007), even when using the same statistical methods of analysis for both datasets. Another strength of the data evaluated in the present study is that the samples were collected as part of the routine care of subjects. Thus, physicians were involved in the collection of the samples, and all the study subjects were previously, independently diagnosed by a physician, using ICD-9 coding. As a result, it is expected that determinations made by physicians in such a setting should not have biased the present data (i.e. the diagnoses were made prior to lab testing and the values determined were used to direct medical care for subjects), and factors influencing willingness of a given subject to voluntarily participate in an experimental study, that may have skewed the results observed by Hertz-Picciotto and coworkers (2010), would not be expected to impact the present study. Further, the results of the present study are strengthened by the statistical robustness of the effects observed, and by the consistency of the findings in biologically plausible directions. The P-values indicate a very low order of probability that the results observed were due to statistical chance and remain significant even when adjusting for potential confounders such as age, gender, and date of sample collection. The consistency and magnitudes of the observations between logistic regression models and threshold modeling support the present results as true effects. The results of the present study are also consistent with significant associations observed between increased blood Hg levels and other neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD) (Cheuk and Wong 2006).

The type of analytical method which was used to analyze blood Hg levels is also a significant strength of the present study. Berglund and colleagues (2005) previously described that blood RBC Hg levels tended to be significantly higher than other measurements of Hg in whole blood or plasma. As a result, the measurement of blood RBC Hg levels in the present study helped to ensure that few samples examined would be impacted by having Hg levels below the limit of detection, which might have adversely impacted the overall distribution pattern of a series of samples analyzed and resulted in an overall skewing of the distribution patterns of data examined. The measurement of blood RBC Hg levels also helped to ensure greater certainty regarding the quantitative measurement of Hg levels present in individual samples. These types of methodological difficulties were apparently encountered by Hertz-Picciotto and coworkers (2010) in their analyses. Hertz-Picciotto and others (2010) described that there was a wide variation and skewed distribution of Hg levels in their study, and further, non-detectable values of blood Hg levels were assigned specific values by the investigators. In contrast, the accuracy of quantifying the Hg present in the blood samples analyzed in the present study was verified by observing comparable blood RBC total Hg levels in split samples sent to Vitamin Diagnostics and the University of Rochester, as shown in Table II.

The main limitation of the present study is that, as a retrospective case-control study, it is impossible to establish a direct causal link between the increased blood Hg levels observed and the diagnosis of an ASD in the subjects examined. Notwithstanding this problem, the consistency of the present results, along with other methods of examination that have previously shown a significant association between Hg exposure and an increased risk of an ASD diagnosis, suggests that Hg exposure is an important causal factor for some individuals diagnosed with an ASD.

Another limitation of the present study is that no information was available regarding the specific sources of Hg exposure which produced the significantly increased blood Hg levels observed among subjects diagnosed with an ASD in comparison to controls. While this information may be important to assessing the exact risk associated with various types of Hg exposure, this limitation does not directly detract from the significant association observed between blood Hg levels and an ASD diagnosis. The
present study also has a limitation that the treatment status of the subjects examined was not known. This may be important because physicians submitting samples may have advised subjects diagnosed with an ASD to either limit exposure to various environmental sources of Hg or to engage in detoxification therapies to help lower Hg body-burden (Curtis and Patel 2008). In either case, the effect would be that such subjects might significantly lower their blood Hg levels, and as a result, bias the observed differences between subjects diagnosed with an ASD and controls toward the null hypothesis.

CONCLUSIONS

The present study indicates that subjects diagnosed with an ASD have, on average, significantly higher levels of Hg in their blood than controls. The neurotoxicity of Hg is well-established, and it is known that even small amounts of Hg can cause neurological injury similar to the brain pathology found in subjects diagnosed with an ASD (Austin 2008, Geier et al. 2008, 2009e). In addition, recent research indicates subjects diagnosed with an ASD have a decreased detoxification capacity for Hg (Geier et al. 2009a). The weight of evidence provided by a variety of different studies offers a compelling argument for the hypothesis that Hg is a causal factor in the neuropathology reported in subjects diagnosed with an ASD.

It is recommended that further research should be conducted to evaluate the consistency of the present results with those in other populations of subjects diagnosed with an ASD. Investigators should also examine the potential correlation between elevated Hg and other potential markers of adverse effects in subjects with an ASD diagnosis, and physicians should consider treatment options that may be available for Hg-intoxication in subjects diagnosed with an ASD.

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Blood mercury levels and autism


