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ORIGINAL ARTICLE

Altered urinary porphyrins and mercury exposure as biomarkers for autism severity in Egyptian children with autism spectrum disorder

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Abstract Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder that affects social, communication, and behavioral development. Recent evidence supported but also questioned the hypothetical role of compounds containing mercury (Hg) as contributors to the development of ASD. Specific alterations in the urinary excretion of porphyrin-containing ring catabolites have been associated with exposure to Hg in ASD patients. In the present study, the level of urinary porphyrins, as biomarkers of Hg toxicity in children with ASD, was evaluated, and its correlation with severity of the autistic behavior further explored. A total of 100 children was enrolled in the present study. They were classified into three groups: children with ASD (40), healthy controls (40), and healthy siblings of the ASD children (20). Children with ASD were diagnosed using DSM-IV-TR, ADI-R, and CARS tests. Urinary porphyrins were evaluated within the three groups using high-performance liquid chromatography (HPLC), after plasma evaluation of mercury (Hg) and lead (Pb) in the same groups. Results showed that children with ASD had significantly higher levels of Hg, Pb, and the porphyrins pentacarboxyporphyrin, coproporphyrin, precoproporphyrin, uroporphyrins, and hexacarboxyporphyrin compared to healthy controls and healthy siblings of the ASD children. However, there was no significant statistical difference in the level of heptacarboxyporphyrin among the three groups, while a significant positive correlation between the levels of coproporphyrin and precoproporphyrin and autism severity was observed. Mothers of ASD children showed a higher percentage of dental amalgam restorations compared to the mothers of healthy controls suggesting that high Hg levels in children with ASD may relate to the increased exposure to Hg from maternal dental amalgam during pregnancy and lactation. The results showed that the ASD children in the present study had increased blood Hg and Pb levels compared with healthy control children indicating that disordered porphyrin metabolism might interfere with the pathology associated with the autistic neurologic phenotype. The present study indicates that coproporphyrin and precoproporhyrin may be utilized as possible biomarkers for heavy metal exposure and autism severity in children with ASD.

Keywords Autism · Mercury exposure · Urinary porphyrins · Toxicity biomarkers

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Introduction

Autism spectrum disorder (ASD) is a multi-factorial and complex neurological disorder, which appears to be affected by environmental, nutritional, immunological, and genetic factors as well as further conditions even related to a high sensitivity to oxidative stress (Yassa 2014; Deisher et al. 2015; Endreffy et al. 2016; Jumah et al. 2016). Environmental agents,

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such as mercury (Hg), lead (Pb), measles, and alcohol abuse in pregnant women have been recently reported to be implicated in the possible etiology of ASD (Lyall et al. 2014; Yoshimasu et al. 2014; Dickerson et al. 2015). Furthermore, fertilizers, chemical waste products, building materials, industrial paint, dental amalgam restorations, and nasal sprays are considered possible sources of heavy metal poisoning, causing ASD (Dickerson et al. 2015; Kern et al. 2015). For example, children may be entered in contact with Pb-containing pollutants in the macroenvironment of seamy, poorly safety macro-environments, such as urban open spaces and roads; likewise, workers, particularly if females, used to be in professional contact with Pb (Dickerson et al. 2015). In ASD children, toxicological problems usually appear to be associated with decreased excretion, rather than high exposure only (Bjørklund 2013).

Mercury has a complex metabolic turnover with different half-lives in different organs. A suitable indicator medium that reflects the amount of Hg in the critical organs (brain and kidneys) do not exist (Bjørklund 1991; Rooney 2013). The toxicity of Hg compounds is particularly related to the harmful effects of Hg on the central nervous system (CNS). Mercury inhibits thiol-sensitive enzymes (Pendergrass et al. 1997), impairs heme synthesis and porphyrin metabolism, leading to neuropsychiatric disorders (Fido and Al-Saad, 2005; Mutter et al. 2005; Lakshmi Prya and Geetha 2011; Albizzati et al. 2012; Yassa 2014). In some children with ASD, elevated blood mercury (BHg) levels may induce neuroinflammation with subsequently increased levels of neurokinin A (Mostafa et al. 2016a).

Even very low levels of Pb exposure may cause toxicological effects with behavioral concomitants (Lidsky and Schneider 2003). Furthermore, Pb induces neuroinflammation and autoimmunity in ASD (Mostafa et al. 2016b). A recent study suggests that increased levels of BPb in some children with ASD may trigger the production of serum anti-ribosomal P antibodies (Mostafa et al. 2016b). Some genetic and environmental factors may increase the harmful effects of Pb on neural development, thus making some children more vulnerable to Pb neurotoxicity (Lidsky and Schneider 2003).

Porphyrins are synthesized in tissues as intermediates in the production of heme; in this pathway, uroporphyrin decarboxylase and coproporphyrinogen oxidase reactions are the most vulnerable enzymatic steps in heme pathway. Mercury-derived toxicity has been associated with increased urinary coproporphyrin, pentacarboxyporphyrin, and precoproporphyrin (Nataf et al. 2006; Wang et al. 2011). The kidney is the primary organ for the excretion and clearance of Hg-containing compounds. Therefore, changes in urinary porphyrin excretion patterns might be used as a specific marker to evaluate Hg tissue accumulation and biological effects, particularly in the kidneys following a prolonged Hg exposure (Wong et al. 2015).

The aim of the present study was to estimate the level of urinary porphyrins as biomarkers of Hg toxicity in ASD children and to explore its correlation with severity of the autistic neuropsychiatric pattern and behavior.

Materials and methods

Subjects

The present study is an observational case-control comparative study, as a preliminary research for a further randomized controlled trial. The study was carried out in an Outpatient Clinic for "Autistic Disorders", Medical Research Center of Excellence, at the National Research Center in Giza, Egypt and approved by the local Ethical Committee, previous informed consent from parents of the recruited pediatric subjects. The present study included three different patient groups: Group I included 40 hospitalized children with ASD, aged 3–6 years, sex equally distributed, previously diagnosed with ASD. Group II included 40 healthy age and sex matched children, aged 3–6 years, without any neuropsychiatric disorder. Group III included 20 healthy normal siblings to the ASD children (Group I) with an age range between 3 and 15 years old.

Children with ASD were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR) criteria (APA 2000), Autism Diagnostic Interview-Revised (ADI-R) (Rutter and Lord 2003), and the Childhood Autism Rating Scale (CARS) (Schopler et al. 1998).

Throughout all experiments, the Ethical Committee of the National Research Center, Giza, Egypt approved the research protocols. Informed consent was also obtained from the parents of the studied subjects for blood withdrawal, urine collections, and consent for research purposes.

Blood sampling and analysis

Each peripheral venous blood sample was collected from each single patient and control in a triplicate way, in order to ensure stability in the process, using EDTA-containing vacuum tubes for Hg and Pb analysis, which was performed within 4 h from blood withdrawal by cold vapor atomic absorption spectrometry (Wang and Hansen 2005). Pre-analytical controls and process were displaced and complied in order to warrant a reduction in bias coming from sampling and handling.

Urine sampling and analysis

Urine was collected according to our labs standard. Urinary porphyrins excretion were estimated by evaluating their presence in urine samples by high-performance liquid chromatography (HPLC) with MS/MS (Tandem mass) spectrometry, using both photodiode array (PDA) and mass detectors (Danton et al. 2006). The instrument response was based on the peak area, which was

Table 1 Age and gender of studied children with ASD, healthy controls, and healthy siblings of the ASD children

Variables	Group I (autistic) no = 40		Group II (controls) no = 40		Group III (siblings[a]) no = 20	
Age (years) Range	3–6		3–6		3–15	
Mean ± SD	4. 118 ± 0. 9383		5. 23 ± 1. 2453		9. 5 ± 5. 9	
Sex	Number	%	number	%	number	%
Male	32	80	28	70	12	60
Female	8	20	12	30	8	40

[a] Healthy siblings of the children with ASD in Group I

calculated with a Xcalibur, Version 1.1. by integration and processing of the peak, according to previously published methods (Stoev et al. 2007).

Statistical analysis

Data were expressed as mean ± SD. Statistical significance was assessed using a Cross Tabulation Test, normality tests (Kolmogorov-Smirnov) and ANOVA with Tukey's post hoc test. A probability value of $p < 0.05$ was considered statistically significant for the null hypothesis (H_0). Bivariate comparisons were examined using Pearson's correlation coefficients for numerical parametric variables.

Results

Stratification of the investigated population is described in Table 1. Following CARS scores, children with ASD were classified into mild autism (CARS score: 30–33), encompassing 20 children (50 %); moderate autism (CARS score: 34–37), encompassing 14 children (35 %); and severe autism (CARS score: > 37), with only six children (15 %).

Of the mothers of the ASD children in Group I, 70 % (28 of 40) had a history of dental amalgam restorations just before pregnancy where their children had moderate to severe form of autism. By weight, dental amalgam fillings contain about 50 % Hg (Kern et al. 2014). No history of dental amalgam restorations was reported in the other groups. When analyzed, urine excretion children with ASD had significantly higher levels of pentacarboxyporphyrin, coproporphyrin, precoproporphyrin uroporphyrins, and hexacarboxyporphyrin when compared with healthy controls ($p < 0.001$, see Table 2). In regard to heptacarboxyporphyrin level, no significant statistical difference was observed between the groups ($p < 0.3$). The results revealed that all urine porphyrins were significantly higher in the children with ASD (Group I) than in their healthy siblings (Group III) (Table 2). Furthermore, the comparison between levels of the porphyrins and the severity of autism showed that the respective mean values of pentacarboxyporphyrin, coproporphyrin and precoproporphyrin were significantly higher in the children with a moderate degree of autism. Further porphyrins showed no significant statistical difference (Table 3). These results showed that there was a significant positive correlation ($p < 0.05$) between the levels of coproporphyrin and precoproporphyrin and CARS, denoting that the severity of ASD increases with the increase in their levels (Figs. 1 and 2).

Moreover, the mean values of both Hg and Pb were significantly higher in the ASD patients (Group I) as compared with the healthy controls and healthy siblings of the children with ASD (Group II and III) (Table 4). There was a significant positive correlation between Hg and pentacarboxyporphyrin,

Table 2 Comparison of urinary porphyrins levels between children with ASD, healthy controls, and healthy siblings of the ASD children

Parameters(μg/L)	Group I (ASD) Mean ± SD	Group II (healthy controls)	Group III (siblings[a])
Uroporphyrins	35. 17 ± 6. 92	23. 72 ± 8. 5	22. 6 ± 7. 11
P-value		*0. 001***	*0. 001***
Heptacarboxyporphyrin	2. 6 ± 1. 09	2. 4 ± 0. 8	1. 7 ± 0. 7
P-value		*0. 3*	*0. 003***
Hexacarboxyporphyrin	2. 99 ± 1. 05	2. 3 ± 0. 6	1. 4 ± 0. 5
P-value		*0. 03**	*0. 001***
Pentacarboxyporphyrin	8. 270 ± 1. 26	3.11 ± 1.18	2. 1 ± 0. 5
P-value		*0. 001**	*0. 001***
Precoproporphyrin	15. 845 ± 4. 3	4.86 ± 29. 25	3. 1 ± 1. 1
P-value		*0. 001**	*0. 001***
Coproporphyrin	297.50 ± 61. 07	88 ± 29. 25	59. 6 ± 22. 4*
P-value		*0. 001***	*0. 001***

[a] Healthy siblings of the children with ASD in Group I

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 3 Comparison between urinary porphyrins levels and the severity of autism

Parameters (μg/L)	Mild no = 20	Moderate no = 14	Severe No = 6	*P* Value * $p < 0.05$
Mean ± SD				
Pentacarboxyporphyrin	7. 7 ± 1. 4	8. 8 ± 0. 8	8. 6 ± 0. 5	0. 03*
Coproporphyrin	271 ± 59. 8	331 ± 59. 3	306 ± 16. 5	0. 01*
Precoproporphyrin	14. 2 ± 4. 1	17. 6 ± 4. 1	17 ± 4. 2	0. 05*
Uroporphyrin	35. 1 ± 7. 5	34. 4 ± 6. 3	36. 8 ± 7	0. 7
Hexacarboxyporphyrin	3. 1 ± 1. 03	2. 5 ± 1. 02	3. 4 ± 1. 06	0. 1
Heptacarboxyporphyrin	2. 4 ± 1. 1	2. 6 ± 0. 7	2. 9 ± 1. 6	0. 6

coproporphyrin, precoproporphyrin, uroporphyrin, and hexacarboxyporphyrin (Figs. 3, 4, 5, 6, 7) and (Table 5). However, the correlation between the level of Hg and heptacarboxyporphyrin showed no statistical significance. A significant positive correlation was observed between the levels of Hg in the blood and the CARS, suggesting that as the level of Hg may increase the severity of autism increases (Fig. 8).

Discussion

The aim of the present study was to estimate blood levels of Hg and the urinary porphyrins as possible biomarkers in children with ASD. The plasma levels of Pb and Hg in ASD children were compared with the values in healthy controls, and healthy siblings of the ASD children, showing that Pb and Hg were significantly higher in the ASD group. This result is consistent with the results of the studies by Alabdali et al. (2014) and Metwally et al. (2015), who reported higher levels of toxic metals such as Pb, Hg, arsenic (As), and aluminum (Al) in children with ASD compared to healthy controls and exploring possible associations between ASD and neurotoxic metals. The reason for the greater body burden of toxic metals in children with ASD may be related to increased exposure to toxic metals, increased absorption as a result of intestinal permeability and decreased capability to excrete toxic metals.

The present study provides new information that plasma Hg (p-Hg) levels correlate with Hg associated porphyrins in ASD and that p-Hg levels correlate with autism severity. The study confirms findings from previous research that shows a Hg association in ASD, including elevated p-Hg levels in ASD and a Hg associated porphyrin pattern in ASD (Geier and Geier 2006, 2007, 2008; Austin and Shandley 2008; Geier et al. 2009a,b; Kern et al. 2010, 2011a,b; Youn et al. 2010).

In the present study, most probably Hg from the dental amalgam fillings of the enrolled mothers should have a predictive potential in the etiopathogenesis or exacerbation of ASD. The present study showed that 70 % of mothers with ASD children had dental amalgam fillings while 30 % (12 of 40) had not. In a recent study a correlation between the number of dental amalgams in the mother and the increased risk of ASD in the offspring was found (Mortazavi et al. 2015).

High residual concentrations of Hg in mothers' dental amalgam may induce a significant DNA corruption in lymphocytes and human tissue cells, increase oxidative stress and reduced glutathione levels (Mutter 2011). In addition, levels of Hg in the brains of infants whose mothers were dental amalgam bearers are sufficient to inhibit the function of methionine synthetase enzyme. Methionine synthetase is crucial for methylation and therefore for brain development,

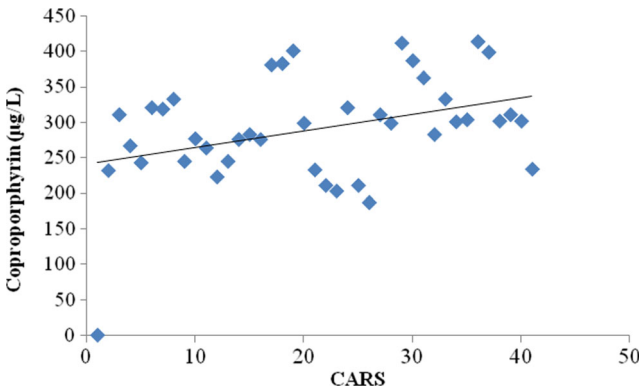

Fig. 1 Pearson correlation test* between Childhood Autism Rating Scale (CARS) (scores) and urine coproporphyrin (μg/L) measured by HPLC and mass tandem spectrometry in ASD subjects. * Paired t-test analysis for the Pearson correlation test $p < 0.01$.

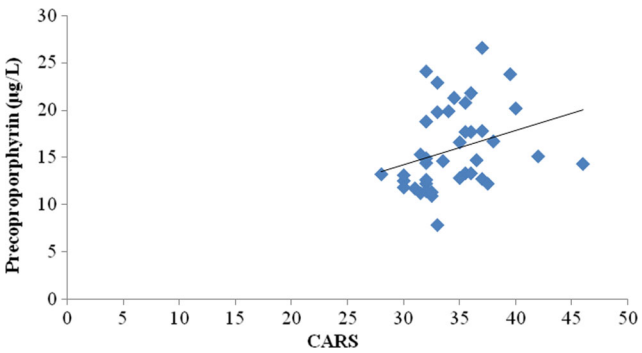

Fig. 2 Pearson correlation test* between Childhood Autism Rating Scale (CARS) (scores) and urine precoproporphyrin levels (μg/L) measured by HPLC and mass tandem spectrometry in ASD subjects. * Paired t-test analysis for the Pearson correlation test $p < 0.001$.

Table 4 Levels of plasma mercury and lead in children with ASD, healthy controls, and healthy siblings of the ASD children

Parameters	Group I (ASD) no = 40 Mean ± SD	Group II (healthy controls) no = 40	Group III (siblings[a]) no = 20	*P*-value
Mercury (μg/L)	32.9 ± 16.4	12.08 ± 4.5	12.08 ± 4.5	<0. 001
Lead (μg/L)	16.4 ± 7.05	10.8 ± 3.4	10.8 ± 3.4	<0. 001

[a] Healthy siblings of the children with ASD in Group I

maturation of nerve cells, and production of neurotransmitters. Maternal amalgam fillings may increase Hg levels in cord blood. The risk for delayed neurodevelopment of children was markedly increased when Hg levels in cord blood were higher than 0.8 ng Hg/mL thus may cause neurodevelopmental deficits of their infants (Mutter 2011).

When the levels of six main porphyrins were measured, the levels of all of them were significantly higher in the ASD children compared to healthy controls except for heptacarboxyporphyrin, which showed no significant difference in its level among children with ASD. Several possibilities might account for these changes in the levels of the porphyrins. One of these possibilities is that the excretion patterns of porphyrins are metal specific in humans after prolonged exposure to Hg and their appearance in unusual patterns suggests the existence of Hg and its extent of body burden (Kern et al. 2015).

Mercury is probably the only toxic metal, known since to date, able to elevate the precoproporphyrin excretion. This is because precoproporphyrin is an atypical urinary porphyrin that is not normally present in the urine, and is formed as a consequence of Hg inhibition of uroporphyrinogen decarboxylase in the kidneys during prolonged exposure, paving the way for producing excess pentacarboxylporphyrinogen, which competes with coproporphyrinogen as a substrate for coproporphyrinogen oxidase and precoproporphyrin formation (Woods et al. 2005). Correlation between the levels of Hg and the different types of

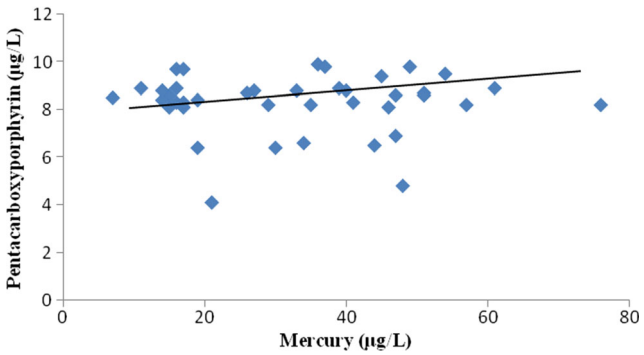

Fig. 3 Pearson correlation test* between blood mercury levels (μg/L) measured by atomic absorption spectrometry and urine pentacarboxyporphyrin (μg/L) measured by HPLC and mass tandem spectrometry in ASD subjects. * Paired t-test analysis for the Pearson correlation test $p < 0.05$.

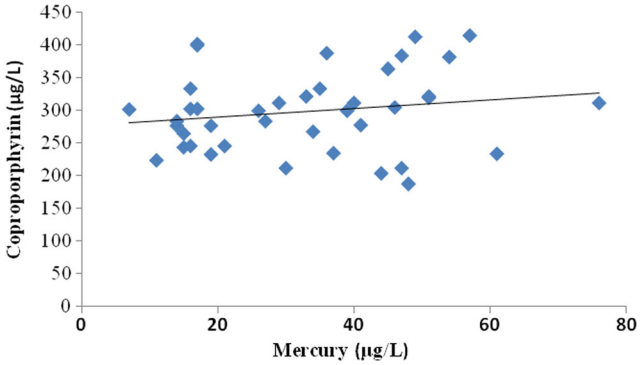

Fig. 4 Pearson correlation test* between blood mercury levels (μg/L) measured by atomic absorption spectrometry and urine coproporphyrin (μg/L) measured by HPLC and mass tandem spectrometry in ASD subjects. * Paired t-test analysis for the Pearson correlation test $p < 0.05$.

porphyrins revealed that there was a significant positive correlation between Hg and pentacarboxyporphyrin, coproporphyrin, precoproporphyrin, uroporphyrin, and hexacarboxyporphyrin. However, there was no significant correlation between the level of Hg and heptacarboxyporphyrin. The present study discovered a positive correlation between the levels of Hg in the ASD children and their CARS scores, which would strongly suggest that increased level of Hg increases the severity of autism.

In the interpretation of the results, it should also be considered that the level of porphyrins in urine could be influenced by other factors than toxic heavy metals. Among others, the disturbance of porphyrins metabolism may be due to haemochromatosis, hepatitis C, HIV disease and poisoning by various organic molecules, including ethanol and hexachlorobenzene (Macedoni-Lukšič et al. 2015).

Regarding the correlation between the levels of different types of porphyrins and the CARS scores among the children with ASD, the results of the present study showed that there was a significant correlation between the levels of coproporphyrin and precoproporphyrin and CARS denoting that the severity of ASD increases with the increase in their

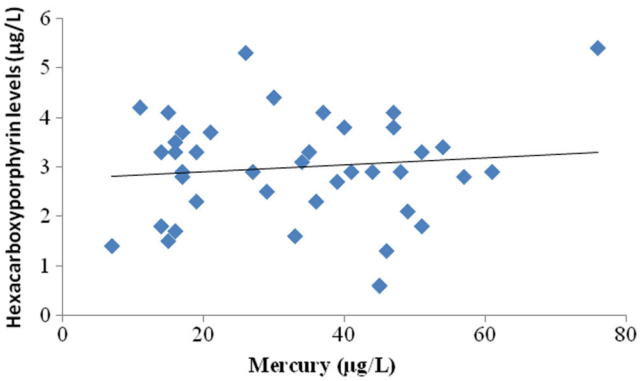

Fig. 5 Pearson correlation test* between blood mercury, levels (μg/L) measured by atomic absorption spectrometry and urine hexacarboxyporphyrin (μg/L) measured by HPLC and mass tandem spectrometry in ASD subjects. * One-sided paired t-test analysis for the Pearson correlation test $p < 0.05$.

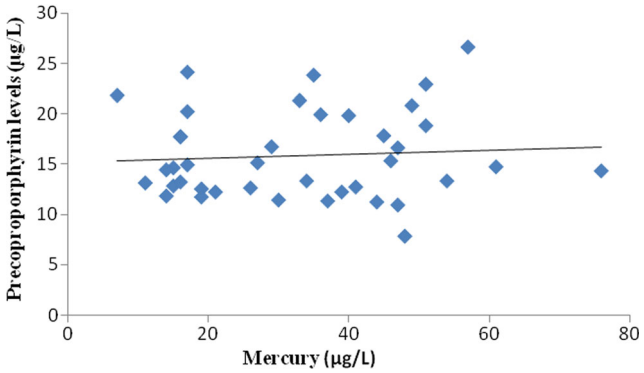

Fig. 6 Pearson correlation test* between blood mercury levels (µg/L) measured by atomic absorption spectrometry and urine precoproporphyrin (µg/L) measured by HPLC and mass tandem spectrometry in ASD subjects. * One-sided paired t-test analysis for the Pearson correlation test $p < 0.05$.

levels. However, there were no significant differences between the further studied porphyrins and the CARS scores. The results of the present study are in partial accordance with a result of Geier et al. (2009c), who examined the relationship between ASD symptom severity using CARS scores and urinary porphyrin levels among the participants.

Heme hypothesis might explain the elevation of urinary porphyrin concentrations in ASD children. Heme has a crucial role as a signaling molecule in synapse development and glutaminergic neuronal receptor processing as well as signaling in the CNS and the regulation of the serotonin (5-hydroxytryptamine) synthesis. Disorders of these systems have been involved as etiological factors in ASD. Because glutaminergic signaling heme has been identified as a regulatory factor in the formation of N-methyl-D-aspartate (NMDA) receptor subunits NR1 and NR2 (Chernova et al. 2011), both are constituents of putative convergent biological pathways comprising the core autism phenotype (Bill and Geschwind 2009). In the serotonin system, heme regulates the activity of

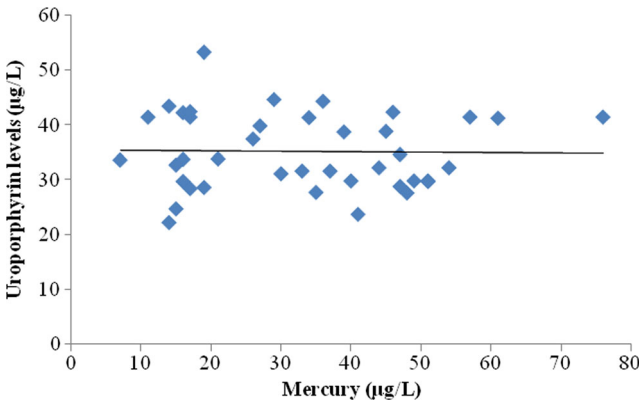

Fig. 7 Pearson correlation test* between blood mercury, levels (µg/L) measured by atomic absorption spectrometry and urine uroporphyrin (µg/L) measured by HPLC and mass tandem spectrometry in ASD subjects. * One-sided paired t-test analysis for the Pearson correlation test $p < 0.05$.

Table 5 Correlation between the levels of mercury and the porphyrins

	Mercury	
	Pearson Correlation	Significance (p)
Hexacarboxyporphyrin	0. 289	0. 009**
Heptacarboxyporphyrin	−0. 073	0. 519
Pentacarboxyporphyrin	0. 601	0. 0001***
Coproporphyrin	0. 653	0. 0001***
Precoproporphyrin	0. 601	0. 0001***
Uroporphyrin	0. 357	0. 001***

** $p < 0.01$; *** $p < 0.001$

tryptophan 2, 3-dioxygenase (TDO2), the rate-limiting enzyme in the catabolism of tryptophan, the immediate precursor of serotonin. Defects in serotonin metabolism as well as abnormalities in circulating and brain serotonin levels have been reported in a broad range of human behavioral and psychiatric disorders including ASD. In particular, increased serotonin has been reported to one-third of children with ASD (Owley et al. 2003).

Conclusion

High Hg levels in children with ASD may relate to increased exposure to Hg, but can also be an effect of decreased ability to excrete Hg, leading to a higher body burden. Coproporphyrin and precoproporhyrin may be utilized as possible biomarkers for heavy metal exposure and severity of autism. The results of the present study indicate that children with ASD have higher blood Hg and Pb levels than healthy control children, and that the higher the level of Hg found in the blood and in body burden (as indicated by the porphyrins) the more severely affected the child is. These findings provide new information as well as corroborate the findings of

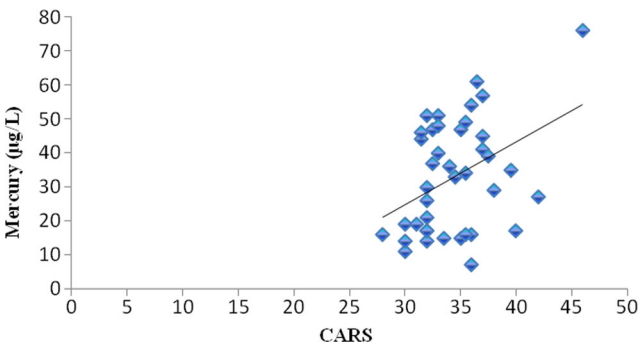

Fig. 8 Pearson correlation test* between Childhood Autism Rating Scale (CARS) (scores) and blood mercury levels (µg/L) measured by atomic absorption spectrometry in ASD subjects. * Two-sided paired t-test analysis for the Pearson test $p < 0.01$.

previous research and suggest that Hg and Pb are causal or contributory in the pathogenesis of ASD.

Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest with respect to the authorship, and/or publication of this article.

Ethical approval All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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