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RESEARCH ARTICLE

The plasma zinc/serum copper ratio as a biomarker in children with autism spectrum disorders

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Abstract

The frequency of zinc deficiency, copper toxicity and low zinc/copper in children with autism spectrum disorders (ASDs) may indicate decrement in metallothionein system functioning. A retrospective review of plasma zinc, serum copper and zinc/copper was performed on data from 230 children with autistic disorder, pervasive developmental disorder-NOS and Asperger's syndrome. The entire cohort's mean zinc level was 77.2 μg dl^{-1}, mean copper level was 131.5 μg dl^{-1}, and mean Zn/Cu was 0.608, which was below the 0.7 cut-off of the lowest 2.5% of healthy children. The plasma zinc/serum copper ratio may be a biomarker of heavy metal, particularly mercury, toxicity in children with ASDs.

Keywords: *Plasma zinc/serum copper ratio; zinc deficiency; metallothionein system dysfunction; mercury; autistic disorder; pervasive developmental disorder-NOS; Asperger's syndrome*

Introduction

Overview

In recent years, much attention has been given to a related group of child neurological disorders called pervasive developmental disorders, which include autistic disorder, pervasive developmental disorder–not otherwise specified (PDD-NOS) and Asperger's syndrome (Lathe 2006). These disorders cause difficulties with production of language, the performance of social skills, and the inhibition of non-functional, repetitive behaviour patterns. Asperger's Syndrome is characterized by impairment in social interaction, inappropriate speech and difficulty controlling obsessive thoughts and behaviours in the setting of normal overall cognitive ability. During the 1970s, the prevalence of autism in the United States ranged from 1 to 3 in 10 000 (Kirby 2005). In the early 21st century the prevalence of all forms of autism has reached 1 in 150 children under 18 (CDC 2007a). The state of New Jersey currently has the highest prevalence of autism in the United States at 1 in 94 births (CDC 2007b). Recent research has sought to determine common characteristics in children with autism spectrum disorders (ASDs). This study describes the status of plasma zinc and serum copper concentrations and the plasma zinc/serum copper ratio in children diagnosed with ASDs.

Role of zinc

Zinc (Zn) has a central role in immune system functioning and individuals deficient in zinc experience an increased susceptibility to various pathogens. Individuals who suffer from severe zinc deficiency often demonstrate severely suppressed immune function, repeated infections and emotional disturbances (Shankar & Prasad 1998). Additionally, zinc provides an essential building block for metal responsive transcription factor-1 (MTF-1). MTF-1 enhances the transcription of metallothionein genes in response to heavy metal load and up- and downregulates numerous genes and enzymes responsible for elimination of heavy metals with potential deleterious effects on mammalian tissue (Wimmer et al. 2005, Wang et al. 2004). Metallothioneins (MTs) are cysteine-containing, low-molecular weight, intracellular proteins with high affinity for metals, being

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the most common intracellular proteins which bind to metals (Andrews 2000). The MT system is essential to heavy metal detoxification throughout the body (Aschner 1996). Zinc is the most efficient MT producer (Park et al. 2001). Zinc is essential to the transcription of MT-I and MT-II present in all vertebrate tissues, including the cerebellum. Increased concentrations of vesicular zinc support MT-III production in the hippocampus, amygdala and pyriform cortex. Zinc promotion of the MTs decreases the neurotoxicity of mercury exposures in rat models and increases the elimination of free radicals by MTs serving as antioxidants (Aschner et al. 2006). Thimerosal, which metabolizes to ethyl mercury, can trigger intracellular zinc release from MT proteins which can be measured by fluorescent signals from lymphocytes (Haase et al. 2008). Mercury compounds may then replace zinc in the binding sites of MTs, leading to eventual removal of mercury from tissues.

The effects of exposing cultured lymphocytes from children with autism and their siblings to thimerosal, containing 49.6% ethyl mercury by weight, and zinc were studied by Walker et al. (2006). Thimerosal exposure caused upregulation of mRNA leading to increases in several heat shock proteins, and 'many of the several hundred upregulated genes represent neurodegeneration and apoptotic pathways, while many of the significantly down-regulated genes belong to metabolic and cell signaling pathways' (Walker et al. 2006). Zinc exposure upregulated several genes that promoted MT protein production.

Variations in the familial transmission of single nucleotide polymorphisms (SNPs) of the metal responsive transcription factor 1 (*MTF1*) gene were found in a recent study by Serajee et al. (2004). The GT haplotype was more frequently transmitted than the GC haplotype. Given the importance of *MTF1* for regulation of MT protein homeostasis in response to heavy metal exposure, these SNPs may be influencing the conformation of MT proteins, adversely affecting the efficient utilization of zinc stores for heavy metal detoxification.

In mammalian models, total body zinc deficiency can be associated with T- and B-cell dysregulation causing dysfunction of the immune system (Shankar & Prasad 1998). During a state of zinc deficiency, T-cell functionality is among the first lost among immune cells (Sprietsma 1999). A recent study noted decreases in splenic size and lymphocyte number in the spleen and thymus in intrauterine growth-restricted mice who experienced zinc deficiency *in utero* (Jenkins et al. 2004). Zinc deficiency can occur *in utero* from malnutrition or from increased exposure to heavy metals. Some mammals have decreased MT functionality, beginning in the early fetal developmental stages (Andrews 2000). *In utero*, mammals who have decreased functionality of their MT system may use up their zinc reserves faster than fetuses with normal MT function. Children who are born with decreased zinc stores remain at risk for zinc deficiency throughout childhood. Zinc deficiency is associated with developmental delays, malabsorption secondary to decreased functioning of intestinal zinc-dependent enzymes, and postnatal immune dysregulation (Walker & Black 2004). Low plasma zinc can also lead to copper toxicity, which can cause liver dysfunction and neurological impairment in children.

Role of copper

Copper (Cu) has many important roles in the body, including participation in mechanisms of cell propagation and growth (Leary et al. 2009). Copper supports an effective immune response through promotion of interleukin-2 production using lymphocytic cells (Arredondo & Nunez 2005). Copper is involved in the synthesis of MT proteins. These proteins have a high attraction for copper, and in times of high copper concentrations, MT levels rise (Arredondo & Nunez 2005). In addition to MT, glutathione peroxidase and superoxide dismutase may be upregulated during times of high copper concentration (Roelofsen et al. 2004). Copper has an important role as a cofactor in many metalloenzymes including cytochrome c oxidase, copper/zinc superoxide dismutase and lysyl oxidase. It affects responsiveness to oxidative stress, oxidative phosphorylation and collagen biosynthesis (Knight et al. 1998). Copper is a cofactor in proteins connected with neurological diseases including amyotrophic lateral sclerosis, Alzheimer's disease and Creutzfeldt–Jacob disease (Roelofsen et al. 2004). Oxidative damage to lipids, nucleic acids and proteins can result from increased copper concentrations (Rosenzweig 2001). Free radical concentrations are increased in tissues that contain larger amounts of transition metals, including copper (Ozdemir et al. 2008). Copper can exist in two different oxidation states, causing copper to be toxic in surplus, promoting the creation of reactive oxygen species (De Bie et al. 2005). Serum copper concentrations increase as physiological stress becomes greater in magnitude because of higher concentration of ceruloplasmin, where most of the copper in serum is located (Best et al. 2004).

Copper homeostasis needs to be controlled very closely, as copper becomes toxic in higher than normal concentrations (De Bie et al. 2005). Copper is incorporated into the bloodstream from the gastrointestinal tract (Schubert 1964). To be excreted from the body, copper needs key contributions from the liver (De Bie et al. 2005). In comparison to other necessary trace elements, very little copper is accumulated in the body and adult humans contain less than 100 mg of stored copper (Turnlund 1998). During infancy, higher copper levels become dangerously toxic due to incomplete liver

function (Arredondo & Nunez 2005). Changes in the concentrations of heavy metals, including zinc and copper, have been noted in numerous neurological disorders, including Parkinson's, Alzheimer's and Huntington's Diseases (Hidalgo et al. 2001). When copper concentrations are not controlled properly, it becomes toxic to the central nervous system and liver, as occurs in Wilson's Disease (Roelofsen et al. 2004).

Zinc to copper ratio

In recent years, efforts have been made to establish normal values and ratios of metals in various human media including serum and plasma, with the aim of determining the ranges which best support mammalian enzymatic activity and health. Zinc and copper have antagonistic effects with respect to each other. A nearly 1:1 ratio of zinc to copper in the serum has been associated with more effective immune responsiveness to infectious agents (Van Weyenbergh et al. 2004). Zinc maintains a balance with copper in blood, and the blood levels of the two tend to be inversely related, with low plasma zinc nearly always being associated with high serum copper. The zinc/copper (Zn/Cu) ratio has been implicated as a rapid method of determining the functional state of the MT system. Lower zinc/copper ratios may reflect total body zinc deficiency and decreased efficiency in eliminating deleterious heavy metals from tissues and blood through the MT system. One of the main functions of MTs is to eliminate metal ions, primarily to maintain zinc and copper homeostasis (Aschner 2006). Since zinc and copper both interact with MT, the Zn/Cu ratio may be seen as a way to determine the state of MT system functioning.

There are several published studies that state that the normal zinc to copper ratio, in children and adults, is close to 1:1. In a recent study, Walsh et al. (2001, 2002) performed measurements of the copper/zinc (Cu/Zn) ratio on autistic, PDD-NOS and Asperger's syndrome patients. The mean Cu/Zn ratio for the 503 autism spectrum patients was 1.63 and for the control patients was 1.15 (Walsh et al. 2001, 2002). When changed to Zn/Cu ratio, this data becomes 0.6135 for autism spectrum patients and 0.87 for the control patients (Walsh et al. 2001, 2002). Additionally, Van Weyenbergh et al. (2004) tested plasma zinc and copper concentrations and Cu/Zn ratios in adults with leishmaniasis and healthy controls. In the control group of this study, it was found that the mean Cu/Zn ratio in plasma was approximately 1 (Van Weyenbergh et al. 2004). Leishmaniasis patients had lower Zn/Cu ratios than controls (Van Weyenbergh et al. 2004). Walsh et al. measured serum copper/plasma zinc ratios in assaultive young males. Walsh found the average serum copper/plasma zinc ratio in controls to be 1.02 while it was 1.40 in the assaultive subjects (Walsh et al. 1997). When changed to a Zn/Cu ratio, the controls' value was 0.98 and the assaultive subjects' value was 0.71. The subjects in this study ranged from 3 to 20 years old with a mean of 9.5 (Walsh et al. 1997). This age range is very similar to the one in the current study. Plasma zinc was noted to be a better measure of zinc status than serum zinc (Walsh et al. 1997).

Several studies suggest that mercury toxicity may be a major cause of MT dysfunction in children with ASDs, which may be reflected in the Zn/Cu ratio. Geier et al. studied urinary porphyrins in children with mild versus severe ASDs as determined by the Childhood Autism Rating Scale (CARS) scoring (Geier et al. 2008a). Urinary porphyrins associated with mercury toxicity displayed significantly greater elevations in the severe ASD group than the mild ASD group. These urinary porphyrins were positively correlated with rising CARS scores and plasma oxidized glutathione levels, indicating that a measure of mercury toxicity was associated with a more severe autistic presentation and oxidative stress. Decreased levels of transsulfuration metabolites, cysteine, reduced glutathione and sulfur were present in children with ASDs compared with normal controls, suggesting that mercury-induced MT dysfunction contributed to decrements in these measures of chemical detoxification.

Multiple previous studies strongly suggest that certain urinary porphyrins, including coproporphyrin and precoproporphyrin, are increased in children with ASDs compared with controls, supporting the presence of mercury toxicity in many children with ASDs (Austin & Shandley 2008, Geier & Geier 2007a, 2006, Nataf et al. 2006).

Multiple publications have shown lower levels of mercury in samples of first-cut hair from children with autism, compared with controls. Adams et al. (2008) found that children with lower levels of mercury in their hair were 2.5 times more likely to demonstrate autism, indicating that the development of autism may be associated with decreased ability to eliminate mercury from the body (Adams et al. 2008). Holmes et al. (2003) also noted lower concentrations of mercury in first-cut hair of children with autism compared with controls and described a pattern of decreasing severity of autism spectrum presentation with increasing levels of hair mercury (Holmes et al. 2003). This study supported the perspective that increasing ability to efflux mercury was associated with less neurophysiological dysfunction in these children. In contrast, a controlled study of toxic trace elements in the hair of Kuwaiti children found significantly higher concentrations of lead, mercury and uranium in children with autism (Fido & Al-Saad 2005). Kuwaiti children may be heavily exposed to mercury from bread containing methyl mercury fungicide, and their environment has been dramatically disrupted by war-related pollution. These conditions may have caused autism in Kuwaiti children secondary to overwhelming

mercury and other toxin exposures, even though they maintained adequate MT functioning as indicated by the efflux of mercury into their hair.

Higher levels of mercury are found in the blood, teeth and chelated urine specimens of children with autism compared with controls (DeSoto & Hitlan 2008, Adams et al. 2007, Bradstreet et al. 2003). A case series of children with regressive autism described by Geier and Geier noted that these children displayed elevated androgen levels, which can adversely affect MT functioning, and evidence of decreased performance of glutathione metabolism necessary for mercury excretion (Geier & Geier 2007b).

Air pollution containing mercury has been strongly related to the incidence of autism in children in San Francisco and in Southeastern Texas (Windham et al. 2006, Palmer et al. 2009). Although the Institute of Medicine (IOM) concluded that there is no relationship between exposure to mercury from vaccines in the form of thimerosal and autism and other neurodevelopmental disabilities, studies continue to be published which provide concern that such exposure may be related to the development of neurological disorders (IOM 2004, Young et al. 2008, Gallagher & Goodman 2008). Thimerosal was present in many Rho (D) immune globulin products given to pregnant Rh-negative women prior to 2002. Geier et al. demonstrated that a cohort of children born prior to 2001 with neurodevelopmental disorders were more frequently born to mothers with Rh negativity compared with controls, than children with neurodevelopmental disorders born after 2001, when thimerosal was taken out of Rho (D) immune globulin products (Geier et al. 2008b).

Given the importance of zinc and copper metabolism for healthy neurological functioning and heavy metal, including mercury, detoxification, blood zinc and copper concentrations were measured in the population of children with forms of autism followed by the Neurodevelopmental Service of The Children's Institute (Pittsburgh, PA, USA). This retrospective study sought to determine the frequency of zinc deficiency, copper toxicity and low plasma zinc/serum copper ratio (under 0.7) in a cohort of children with ASDs, which may indicate increased risk for decrement in MT system functioning and malabsorption created by low intestinal zinc-dependent enzyme concentration.

Methods

A retrospective review was performed on data from 230 children (179 male, 51 female, mean age 6.3, standard deviation (SD) of 3.67) with autistic disorder, PDD-NOS and Asperger's syndrome diagnosed by structured interview, play observation and rating scales (including the Childhood Autism Rating Scale and the Asperger's Syndrome Diagnostic Scale) (Schopler et al. 1988, Myles et al. 2001). Diagnostic and Statistical Manual-IV (DSM-IV) criteria were met for these disorders and all children were placed in their diagnostic categories by S.F. post-review of the range of data acquired by S.F. and other evaluating clinicians, which often included Autism Diagnostic Observation Scale scores (Lord et al. 2000). When a rare disagreement about diagnostic classification occurred among involved clinicians, S.F. utilized serial observations during follow-up visits to establish the diagnoses utilized in this study.

This retrospective review of S.F.'s patient population was approved by the Research Committee of The Children's Institute and the Duquesne University Institutional Review Board. Plasma zinc, serum copper and plasma zinc/serum copper ratios were recorded for children with an autism spectrum diagnosis seen by the Neurodevelopmental Pediatrics Service from July 2004 to December 2006, where this information was available. The testing was performed by the University of Pittsburgh Laboratory and Quest Labs (Pittsburgh, PA, USA). The Associated Regional and University Pathologists, Inc. (ARUP) national reference laboratory manual states that the low plasma zinc level for children is 66 $\mu g\ dl^{-1}$, and the high serum copper cut-off is 153 $\mu g\ dl^{-1}$ (Ashwood 2004). An Excel spreadsheet was created to determine the mean, SD and confidence intervals from the data. Analysis of variance (ANOVA) studies were performed using the S Plus program. Only the first plasma zinc and serum copper values obtained after the initial diagnostic visit were recorded. None of the children were on supplements or restrictive diets at the time of the collection of the initial blood work.

Recognizing a Zn/Cu ratio value that generally separates healthy from affected subjects can be accomplished through examination of the normal control groups in two of Walsh's cohorts (Walsh et al. 1997, 2002). In these separate studies, the values of 0.613 and 0.725 were identified to be exactly 2 SD below the mean Zn/Cu ratio of healthy subjects. Through clinical assessment and the values reported in both papers from Walsh et al., a Zn/Cu ratio of 0.7 was chosen as a cut-off. The 0.7 value approximates the cut-off value separating the upper 97.5% from the lower 2.5% of the healthy population, provided Walsh's data is approximately normal.

Results

Analyses were performed to study the entire group as a whole, followed by parcellation of the data into three subgroups: autistic disorder, PDD-NOS and Asperger's syndrome, to determine if significant differences emerged

Table 1. Mean plasma zinc, mean serum copper and mean plasma zinc/serum copper ratio including number of patients in each group. The 12 groups include the entire cohort, separated by diagnosis, gender, and diagnosis and gender. All confidence intervals are taken at the 95th percentile.

	Plasma Zn (μg dl^{-1})	Serum Cu (μg dl^{-1})	Zn/Cu ratio	Number of patients
Entire data set	77.21 ± 1.87	131.37 ± 3.32	0.608 ± 0.020	230
All autistic disorder patients	76.89 ± 3.18	129.68 ± 6.57	0.619 ± 0.037	78
All PDD-NOS patients	77.07 ± 2.59	135.57 ± 4.37	0.585 ± 0.028	110
All Asperger's syndrome patients	78.17 ± 5.36	124.41 ± 7.45	0.644 ± 0.049	42
Male autistic disorder patients	77.26 ± 3.70	132.10 ± 6.62	0.607 ± 0.041	63
Female autistic disorder patients	75.31 ± 6.36	119.49 ± 21.22	0.667 ± 0.084	15
Male PDD-NOS patients	77.66 ± 2.92	134.19 ± 5.09	0.597 ± 0.034	82
Female PDD-NOS patients	75.35 ± 5.85	139.59 ± 8.88	0.555 ± 0.054	28
Male Asperger's syndrome patients	78.67 ± 6.48	125.50 ± 8.60	0.644 ± 0.058	34
Female Asperger's syndrome patients	76.06 ± 8.26	119.75 ± 17.81	0.648 ± 0.101	8
All male patients	77.71 ± 2.17	131.81 ± 3.61	0.610 ± 0.023	179
All female patients	75.45 ± 3.71	130.57 ± 8.25	0.602 ± 0.041	51

PDD-NOS, pervasive developmental disorder–not otherwise specified.

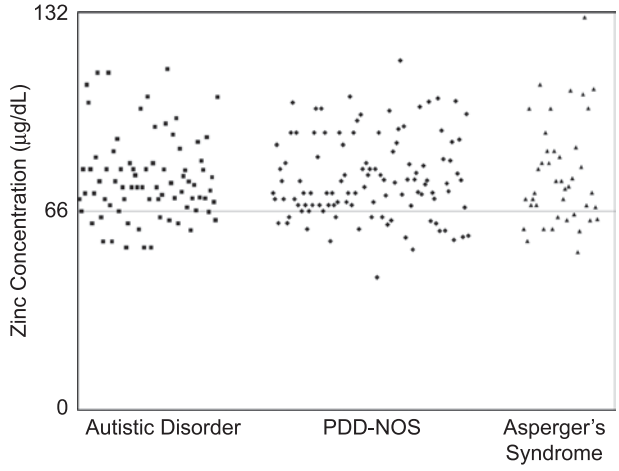

Figure 1. The plasma zinc concentrations for each patient in the cohort. The line at 66 μg dl^{-1} represents the low cut-off value according to ARUP (Ashwood 2004).

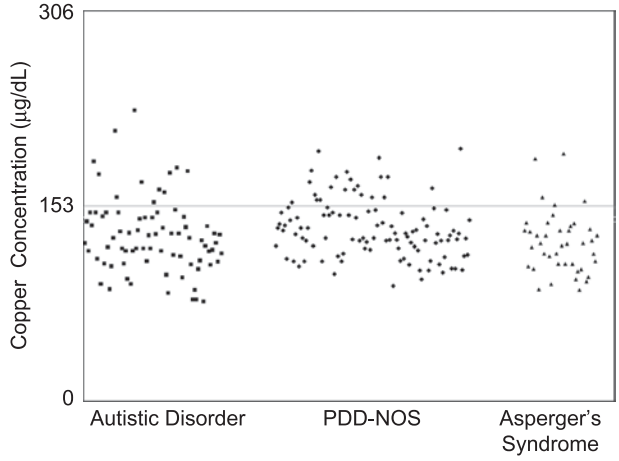

Figure 2. The serum copper concentrations for each patient in the cohort. The line at 153 μg dl^{-1} represents the high cut-off value according to ARUP (Ashwood 2004).

in mean zinc levels, mean copper levels or zinc/copper ratios among the three groups using ANOVA.

Entire cohort

Forty-seven children (20.4%) out of the entire group of 230 children with all forms of autism had low zinc levels below 66 μg dl^{-1}. One hundred seventeen children (50.8%) of the 230 had zinc levels in the lowest 10% (below 75 μg dl^{-1}) of the normal range. The mean zinc level of the 230 children was 77.2 μg dl^{-1} (minimum (min): 44 μg dl^{-1}, maximum (max): 130.4 μg dl^{-1}, SD: 14.5 μg dl^{-1}).

Thirty-nine children (17%) of 230 had high copper levels over 153 μg dl^{-1}. Seventy children (30.4%) of 230 had copper levels in the highest 10% (over 140 μg dl^{-1}) of the normal range. The mean copper level of the 230 children was 131.5 μg dl^{-1} (min: 78.3 μg dl^{-1}, max: 228 μg dl^{-1}, SD: 25.7 μg dl^{-1}).

One hundred sixty seven out of 230 children (72.6%) had zinc/copper ratios under 0.7. The mean zinc/copper ratio of all of the children was 0.608 (min: 0.25, max: 1.01, SD: 0.16). Male and female subjects did not significantly differ in mean zinc (p-value = 0.326), mean copper levels (p = 0.762) or in Zn/Cu ratios (p = 0.774) (Table 1 and Figures 1–4).

Children diagnosed with autistic disorder

The 78 children with autistic disorder had a mean zinc level of 76.9 μg dl^{-1} (min: 54 μg dl^{-1}, max: 113.2 μg dl^{-1}, SD: 14.1 μg dl^{-1}). Fifteen children (19.2%) had low zinc levels below 66 μg dl^{-1}. Forty-one children (52.6%) had zinc levels in the lowest 10% (below 75 μg dl^{-1}) of the normal range.

Their mean copper level was 129.7 μg dl^{-1} (min: 78.3 μg dl^{-1}, max: 228 μg dl^{-1}, SD: 29.1 μg dl^{-1}). Twelve children (15.4%) had high copper levels over 153 μg dl^{-1}.

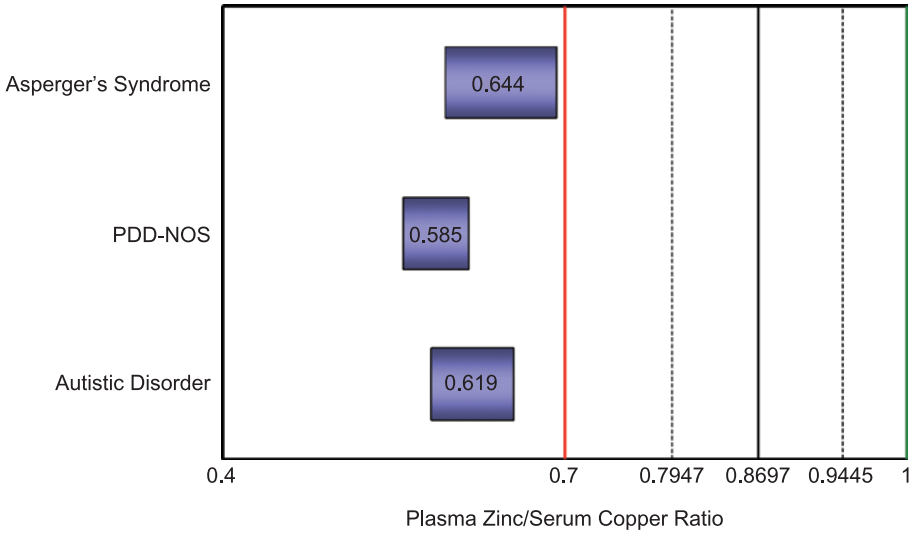

Figure 3. The mean plasma zinc/serum copper ratio and confidence intervals (95%) for Asperger's syndrome, pervasive developmental disorder-not otherwise specified (PDD-NOS) and autistic disorder patients. The confidence interval lengths are represented by the purple bars. The mean values for each group are inscribed within each bar. The black vertical line at 0.8697 is the control mean value from the Walsh et al. study (Walsh et al. 2002). The dotted lines (0.7947 and 0.9445) represent the 95% confidence limits from the Walsh study. The red line at 0.7 represents the cut-off line for a significantly low (bottom 2.5%) plasma zinc/serum copper ratio. The green line at 1 represents the normal value for zinc/copper ratio in children.

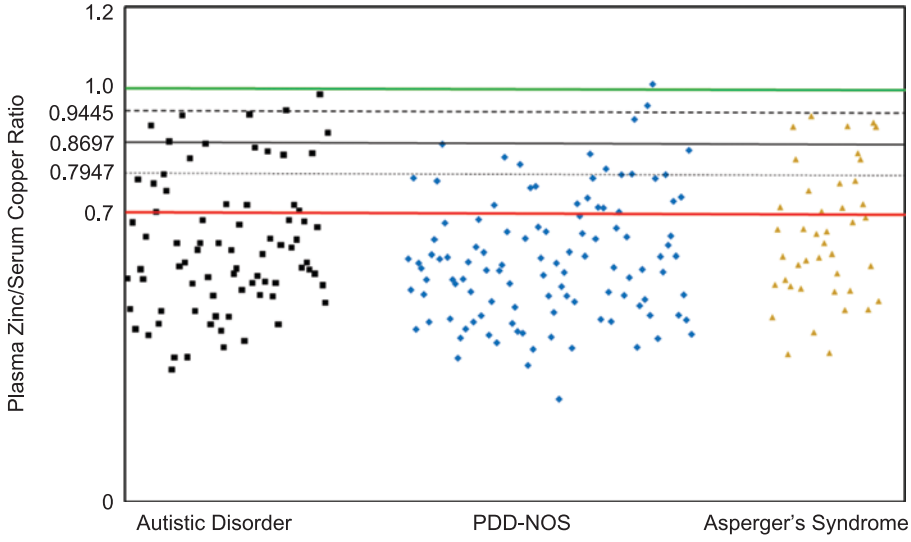

Figure 4. The plasma zinc/serum copper ratio for each individual patient separated by disease. Reference values and intervals are the same as in Figure 3.

Twenty-four children (30.8%) had copper levels in the highest 10% (over 140 μg dl^{-1}) of the normal range.

This subgroup's mean zinc/copper ratio was 0.618 (min: 0.32, max: 0.99, SD: 0.1628). Fifty-six children (71.8%) had zinc/copper ratios under 0.7 (with one at 0.7). Male and female children with autistic disorder did not significantly differ in mean zinc ($p = 0.6324$), copper levels ($p = 0.1324$) or in zinc/copper ratios ($p = 0.2093$) (Table 1 and Figures 1–4).

Children diagnosed with PDD-NOS

The 110 children with PDD-NOS had a mean zinc level of 77.1 μg dl^{-1} (min: 44 μg dl^{-1}, max: 116 μg dl^{-1}, SD: 13.7 μg dl^{-1}). Twenty-one children (19.1%) had low zinc levels below 66 μg dl^{-1}. Fifty-four children (49.1%) had zinc levels in the lowest 10% (below 75 μg dl^{-1}) of the normal range.

The mean copper level was 135.6 μg dl^{-1} (min: 90.7 μg dl^{-1}, max: 197.9 μg dl^{-1}, SD: 23.1 μg dl^{-1}).

Twenty-two children (20%) had high copper levels over 153 μg dl^{-1}. Forty children (36.4%) had copper levels in the highest 10% (over 140 μg dl^{-1}) of the normal range.

This subgroup's mean zinc/copper ratio was 0.587 (min: 0.25, max: 1.01, SD: 0.15). Eighty-four children (76.4%) had zinc/copper ratios under 0.7 (one at 0.7). Male and female children with PDD-NOS did not significantly differ in mean zinc ($p=0.4433$), copper levels ($p=0.2872$) or in zinc/copper ratios ($p=0.1995$) (Table 1 and Figures 1–4).

Asperger's syndrome patients

The 42 children with Asperger's disorder had a mean zinc level of 78.2 μg dl^{-1} (min: 52.3 μg dl^{-1}, max: 130.4 μg dl^{-1}, SD: 17.2 μg dl^{-1}). Eleven children (26.2%) had low zinc levels below 66 μg dl^{-1}. Twenty-two children (52.4%) had zinc levels in the lowest 10% (below 75 μg dl^{-1}) of the normal range.

The children's mean copper level was 124.4 μg dl^{-1} (min: 87.5 μg dl^{-1}, max: 194 μg dl^{-1}, SD: 23.9 μg dl^{-1}). Five children (11.9%) had high copper levels over 153 μg dl^{-1}. Six children (14.3%) had copper levels in the highest 10% (over 140 μg dl^{-1}) of the normal range.

This subgroup had a mean zinc/copper ratio of 0.644 (min: 0.36, max: 0.93, SD: 0.157). Twenty-seven children (64.3%) had zinc/copper ratios under 0.7 (one at 0.7). Male and female children did not significantly differ in mean zinc ($p=0.705$) or copper ($p=0.5472$) levels or in zinc/copper ratios ($p=0.9374$) (Table 1 and Figures 1–4).

Comparison of cohort groups

An ANOVA test for equality of the mean zinc/copper ratio across the three groups was significant at the 10% level; multiple comparisons revealed a significant difference only between the mean zinc/copper ratio of the children diagnosed with PDD-NOS and Asperger's syndrome patients, with the PDD-NOS children having the lower mean ratio ($p=0.03574$). However, a Kruskal–Wallis test for equality of mean age across groups was found to be significant at the 1% level ($p=2.2\times10^{-16}$); multiple comparisons reveal the mean age of the Asperger's syndrome group to be greater than both the PDD-NOS group ($p=2.38\times10^{-8}$) and the autistic disorder group ($p=0.00036$). Zinc/copper ratio was significantly positively correlated with collection age ($r=0.375$, $p=5.24\times10^{-9}$), and regression analyses show disease category to be a non-significant predictor of the zinc/copper ratio in the presence of age. Therefore, the difference in mean zinc/copper ratios among the groups cannot be attributed solely to disease type at this time.

Some significant differences were noted in mean plasma zinc levels and serum copper levels among all three groups. PDD-NOS patients demonstrated higher copper levels on average than children with Asperger's syndrome. The *p*-value when the mean copper values for the PDD-NOS and the Asperger's syndrome children were compared pair wise was 0.009. There was no significant difference found when the mean copper concentrations for PDD-NOS patients were compared with the autistic disorder group ($p=0.1241$) or when the autistic disorder group was evaluated against the Asperger's Syndrome patients ($p=0.317$). When all three group's mean copper concentrations were compared, there was a significant difference found ($p=0.041$).

When the mean zinc concentrations of each of the three groups were evaluated, there was no significant difference found ($p=0.89$). There were no significant differences found when each of the group's mean zinc concentrations were compared: PDD-NOS vs. autistic disorder ($p=0.9267$), autistic disorder vs. Asperger's syndrome ($p=0.6599$), and PDD-NOS vs Asperger's syndrome ($p=0.682$). The autistic disorder group contained the only two children with very elevated serum copper levels over 200 μg dl^{-1}.

Overall, 221 out of 230 patients (96.1%) had zinc concentrations below 105 μg dl^{-1} (the midpoint of normal zinc concentration) with 20.4% below the lower bound of the normal range. For copper, 223 out of 230 children (96.9%) had concentrations above 90 μg dl^{-1} (the midpoint of normal copper concentration) with 17% above the upper bound of the normal range. For zinc/copper ratio, 229 out of 230 patients (99.6%) had a ratio below 1.0 and 170 out of 230 (73.9%) had a zinc/copper ratio at or below 0.7.

Discussion

Children with ASDs from Western Pennsylvania, West Virginia and Southeastern Ohio, frequently demonstrated low plasma zinc/serum copper ratios compared with previously determined norms from the literature (Van Weyenbergh et al. 2004, Walsh et al. 1997, 2001, 2002). In addition, their plasma zinc/serum copper ratios frequently fell below 0.7, a demarcation line that was chosen to represent a significant difference from the near 1:1 ratios seen in previous studies on child and adult controls. The plasma zinc/serum copper ratio for the entire cohort of the present study was 0.608, a figure that was quite similar to the ratio, 0.6135, noted on a previous dataset presented by Walsh et al. in children with ASDs (Walsh et al. 2001, 2002).

Twenty percent of the entire cohort of children with autism spectrum disorders was zinc deficient and 17% of the cohort displayed copper toxicity based on normal ARUP Laboratory ranges. The autistic disorder group contained two children with very high copper levels and

extremely low zinc/copper ratios. Extreme elevations of serum copper may indicate extraordinary stress on the MT system and may have contributed to the severity of these children's presentations. The Asperger's syndrome and PDD-NOS groups did not contain any children with this severity of copper toxicity. Zinc deficiency and copper toxicity contribute to dysregulated neurotransmitter system functioning, decreased zinc finger protein activity and diminished zinc-dependent gastrointestinal enzymatic activity. The activation of the glutamatergic neurotransmitter system has been shown to require zinc uptake to be triggered (Takeda et al. 2006). Synaptic neurotransmission needs zinc as an intracellular signal factor with its involvement with numerous proteins (Takeda et al. 2006). Low zinc concentrations can lead to oxidative stress which can change zinc finger motifs involved as transcription factors in cell signalling (Herbein et al. 2006). Zinc ions control, either directly or indirectly, the molecular structures of zinc finger proteins (Sprietsma 1999). Previous literature showed zinc finger DNA repair proteins could be inhibited by many toxic metals including copper (Asmuss et al. 2000). Copper and other heavy metals prevent the activation of metal-responsive promoters (Heuchel et al. 1994).

There were no significant differences between the zinc/copper ratios of males versus females in the overall cohort ($p = 0.7744$) or in the autistic disorder ($p = 0.2093$), PDD-NOS ($p = 0.1995$) and Asperger's syndrome ($p = 0.9374$) groups. Autistic children appear to demonstrate a similar degree of stress on their MT systems, regardless of their sex. The 3.53 to 1 ratio of males to females in the study may be partially caused by sex differences in metalloprotein functioning, as even very low dose prepubertal estrogen production in human females has been noted to have a salutary effect on metalloprotein production and performance (Chen et al. 2003). There was a significant, positive correlation between zinc/copper ratio and age of collection ($r^2 = 0.14$), which may indicate improved MT functioning in this study's children as they age.

Zinc/copper ratios averaged below 0.7 in the autistic disorder, PDD-NOS and Asperger's syndrome subgroups. These groups, with significantly different behavioural phenotypes, may share some degree of common aetiology involving different levels of MT system dysfunction. The conclusions of this study are limited by the selection of a control group taken from the literature. Nonetheless, several previous studies suggest that a plasma zinc to serum copper ratio of about 1:1 is normative in children and adults (Van Weyenbergh et al. 2004, Walsh et al. 1997, 2001, 2002.). The children with ASDs in this study averaged a plasma zinc to serum copper ratio of 0.608, suggesting a very significant difference in MT functioning compared with healthy subjects. This value is below the 0.7 demarcation line indicating the lowest 2.5% of zinc/copper ratios determined from the Walsh et al. studies (Walsh et al. 1997, 2002).

Western Pennsylvania has been noted to be in the high heavy metal pollution zone of the United States according to data from the Environmental Protection Agency of the USA. Pittsburgh is located in a region of the country where industry is responsible for 73% of the total atmospheric mercury emissions (Gbor et al. 2006). Published studies have shown links between exposure to airborne mercury emissions from coal-fired power plants and increased autism rates (Palmer et al. 2006, 2009). Stress on the MT system of humans triggered by heavy metal exposure may cause faster use of zinc reserves. This cohort's overall propensity for demonstrating decreased zinc levels, elevated copper levels and low zinc/copper ratios may be reflective of exposure beginning *in utero*, and may be affected by their mother's lifetime exposure to ambient heavy metals from the environment.

Zinc's ability to upregulate MT gene expression and reduce toxicity from heavy metal exposure suggests that the provision of zinc to children with ASDs may be an important component of a treatment protocol, especially in children with zinc deficiency (plasma zinc under 66 $\mu g\ dl^{-1}$ according to ARUP) or Zn/Cu ratio under 0.7 (Ashwood 2004).

The plasma zinc/serum copper ratio may be a biomarker that indicates stress on the MT system of children with ASDs. Children with ASDs, according to data from the studied cohort, appear at risk for zinc deficiency, copper toxicity, the demonstration of low zinc/copper ratio, and decreased MT-system functioning. The results of this study suggest that further work is required to explore MT dysfunction and zinc/copper metabolism in children with forms of ASDs.

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