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# Age-dependent lower or higher levels of hair mercury in autistic children than in healthy controls

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An association between autism and early life exposure to mercury is a hotly debated issue. In this study, 91 autistic Polish children, male and female, 3-4 and 7-9 years old, were compared to 75 age- and sex-matched healthy children with respect to: demographic, perinatal, clinical and developmental measures, parental age, birth order, morphometric measures, vaccination history, and hair mercury content. In demographic and perinatal measures there were no consistent differences between the autistic and control groups. Autistic children had a significantly greater prevalence of adverse reactions after vaccinations and abnormal development than controls. Between 45 and 80% of autistic children experienced developmental regress. Autistic children significantly differed from healthy peers in the concentrations of mercury in hair: younger autistics had lower levels, while older – higher levels than their respective controls. The results suggest that autistic children differ from healthy children in metabolism of mercury, which seems to change with age.

Key words: autism, mercury, hair, thimerosal, vaccines, development

Abbreviations: THIM - thimerosal

## INTRODUCTION

Autism spectrum disorders (ASDs) represent a group of neurodevelopmental disorders typified by impairments in verbal and non-verbal communication, social withdrawal and stereotypical behaviors, which may or may not be associated with cognitive deficits, self-injurious behaviors and other neurological comorbidities. The current world-wide epidemic of ASDs and other neurodevelopmental disorders, including attention deficit hyperactivity (ADHD), learning disabilities, and mental retardation constitute the most disturbing public health problems (Robison et al. 1999, Merrick et al. 2004, Altarac and Saroha 2007, Shayer et al. 2007, Hertz–Picciotto and Delwiche 2009). Its magnitude is best illustrated by a dramatic rise in incidences of ASDs in the past 25 years. In many countries current ASDs prevalence is about 1 in 100, whereas in the 1970s and early 1980s it was about 1 in 2500-3000 (Merrick et al. 2004, Baird et al. 2006, Gillberg 2009). It is estimated that only about 5% of the autistic population carries identifiable genetic /chromosomal defects (Newbury et al. 2009). The increase of ASDs prevalence cannot be fully explained by advances in diagnostics or sudden genetic shifts. There is a growing consensus among scientists and clinicians that ASDs ensue from an interaction between biological vulnerability factors and environmental or iatrogenic insults (James et al. 2006, Gillberg 2009).

The contemporaneous emergence of the ASDs epidemic and the introduction of several new infant vaccines in the late 1980s and the 1990s, generated a suspicion that these events might be linked. One of the agents suspected in autism etiology is an organomercury compound, thimerosal (THIM; sodium ethylmercurithiosalicylate containing approximately 49%

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of Hg by weight), which has been used as a vaccine preservative for decades without being comprehensively tested for its safety in developing organisms. A large body of research and at least two centuries of human experience show that all forms of mercury are highly toxic to vertebrates. In the body THIM is metabolized to ethylmercury and then into inorganic mercury compounds (Qvarnstrom et al. 2003). Significant amounts of mercury have been measured in the blood of infants after inoculations with THIM-containing vaccines, with premature infants accumulating over 3 times more mercury than the mature ones (Stajich et al. 2000, Pichichero et al. 2008). Studies conducted with infant monkeys injected with THIM-containing vaccines showed that a few days after vaccinations, concentrations of mercury in the brain were several times higher than those in blood. Mercury levels in the brain may remain markedly increased for many months or years, considering continuous re-exposure to vaccines (Burbacher et al. 2005). Mid-nanomolar concentrations of mercury, which are likely to be reached in the infant brain after inoculation with THIM-containing vaccines, are neurotoxic (Parran et al. 2005, Yel et al. 2005).

The preclinical study of Hornig and coauthors (2004) documented multiple neurodevelopmental disturbances in mice prone to autoimmune diseases after exposure to THIM doses analogous to those used in pediatric vaccines. Our recent study showed that administration of similar doses of THIM to suckling rats causes persistent disruption of endogenous opioid system (Olczak et al. 2009), which resembles opioid dysfunction in autism (Sandyk and Gillman 1986, Sandman 1988). Based on numerous analogies of biological and clinical abnormalities associated with mercury poisoning and autism, the hypothesis emerged linking this disorder with early life exposure to mercurials (Bernard et al. 2001, Mutter et al. 2005). Some epidemiological and ecological studies associated autism and other neurodevelopmental disorders with THIM present in infant vaccines (Geier and Geier 2003, 2006, Young et al. 2008, Gallagher and Goodman 2008, 2009). Other studies denied such a link (Hviid et al. 2003, Madsen et al. 2003), but they were criticized for flawed design and clear conflict of interests (Mutter et al. 2005, Isaacs 2010).

Measurement of heavy metal content in hair is often used as a marker of exposure, because it correlates with past blood levels (Clarkson 1993, Magos and Clarkson 2008). As a non-invasive procedure, it is especially useful for testing children. A few studies compared mercury levels in hair of autistic and healthy children, reporting divergent results. Holmes and coauthors (2003) and Adams and colleagues (2008) demonstrated significantly lower levels of mercury in first baby haircuts of American children diagnosed with autism, than in healthy controls, which was interpreted as possibly impaired mercury and other toxin elimination by autistic children. Reduced levels of heavy metals such as arsenic, cadmium, lead and mercury in hair of autistic children 1-6 years old were also measured by Kern and others (2007). In contrast, strikingly higher levels of hair mercury in autistic Kuwaiti boys (4 to 7 years old) than in healthy controls were detected by Fido and Al-Saad (2005). Higher concentrations of this metal were also found in baby teeth and blood of autistic children than in controls (Adams et al. 2007, Desoto and Hitlan 2007).

Although several studies point to a link between autism and mercury exposure (Mutter et al. 2005, Geier and Geier 2006, Windham et al. 2006, Young et al. 2008, Palmer et al. 2009), the critical sources of this metal and its role in autism pathogenesis are a subject of hot debates, particularly in reference to possible iatrogenic effects of THIM from vaccines and mercury from amalgam fillings. Continuing controversy and public apprehension regarding this issue impelled us to conduct our own study in Polish children, who continue to be inoculated with THIM-containing vaccines. We were particularly interested in identifying possible demographic, perinatal and/or vaccination-related factors, which distinguish autistic from healthy children. In order to assess them we analyzed several birth-related measures such as Apgar scores, body weight, head and chest circumference, Rh conflict, as well as abnormal development in autistic and healthy children of both sexes from two age groups (3-4 and 7-9 years old). We also compared their history of vaccinations, adverse reactions to them, and levels of mercury in hair.

## METHODS

### Study Participants

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association. The protocol was approved by the Ethics

Committee for Human Studies at the Institute of Psychiatry and Neurology. Participation in the study was voluntary. The parents of participants read and signed informed consent forms after the study procedures had been fully explained to them.

Autistic and control children of both sexes from two age groups, 3-4 and 7-9 years old were enrolled into the study. The subjects were not compensated for their participation. The autistic participants were recruited from the children earlier diagnosed with autism in outpatient clinics in Poland. Healthy control children were recruited from 6 preschools and 3 primary Warsaw schools. Recruitment took place from November 2007 to April 2009. All participants were Caucasians, about 40% of autistic children were from Warsaw metropolitan area, others were from different, mostly urban, but some from rural regions located at distances less than 180 km from Warsaw. The autistic children had to fulfill DSM IV criteria for autistic disorder, and score at least 30 points in the CARS scale (see later). Participants were excluded if they had: a neurological and psychiatric disorder other than autism and comorbid disorders; history of liver, renal or endocrine disorder; current infection of respiratory tract or fever state of any origin; and mental retardation. Mental retardation or behavioral disorders, including hyperkinetic disorder in children over 6 years old or significant symptoms of hyperactivity, impulsiveness or restlessness in younger children were exclusion criteria only for the group of healthy control children, but were allowed as comorbid condition in the autistic cohort. Children diagnosed with Asperger's syndrome were excluded from the study. Study participants were divided into two groups. Group I was male and female, autistic and control children 3-4 years old, and group II – similar children 7-9 years old.

### Clinical evaluation

Each autistic child was once more diagnosed by a group of three specialists (2 psychologists and one child psychiatrist), who had over five years experience in autism diagnosis. The examination consisted of semi-structured interview with parents and 1 hour observation of the child's behavior. Extensive medical histories of the autistic and control children were taken. The parents were asked about: detailed history of pregnancy and labor, mental and motor development of the child, any illnesses and traumatic events, history of vaccinations, the occurrence of vaccine-associated adverse effects (if present, parents were asked about their subjective appraisal of observed symptoms and about results of medical professional consultations). Additional information about birth morphometric measures, Apgar scores, vaccinations and development was taken from Child Health Notebooks, which every child born in Poland receives at the hospital and which carries his/her health information until the age of 18 years. The parents of autistic children were also questioned about the first symptoms of autism, which occurred in their child, and the results of previous diagnostic tests. The clinical evaluation of autistic children was based on a one-hour observation of their behavior by two experienced psychologists. The diagnosis of autism was made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM–IV) criteria for autism or pervasive developmental delay (PDD) by a trained professional. The activity and functioning of an autistic child was also assessed according to Childhood Autism Rating Scale (CARS) (Schopler et al. 1980) and the Clinical Global Impression Scale. Children who scored 30 or more points in CARS were diagnosed as autistic.

All control study participants were assessed with use of the Abbreviated Parent-Teacher Questionnaire (IOWA-Conners; version for scientific research (Conners 1969, Rowe and Rowe 1997) in order to exclude children with symptoms of ADHD (diagnosis was made according to DSM IV criteria).

### Measurement of mercury concentration in hair

Haircut samples of autistic and control study participants were obtained from occipital area of head, from the proximal (up to 3 centimeters from scalp) part of hair. The blinded analysis of mercury content in hair by atomic absorption spectrometry was performed at the Chemical Laboratory of Multi-Elemental Analyses at Wroclaw University of Technology. A single-purpose atomic absorption spectrometer based on in situ dry washing followed by gold amalgamation cold vapor AAS method was used for analysis using an Advanced Mercury Analyzer (AMA-254, ALTEC, Czech Republic). This cold vapor AAS method is one of the most widely used techniques for determination of trace amounts of total mercury in environmental materials.

Table I

Demographic data on study participants

| Demographics | Males | | Females | | *p* (*t*-Student, *U*-M-W or $\lambda^2$) |
|---|---|---|---|---|---|
| GROUP I (age 3-4 years) | Autistic | Control | Autistic | Control | |
| N | 30 | 19 | 25 | 19 | |
| Mean age | 3.6 ± 0.1 | 3.5 ± 0.1 | 3.8 ± 0.1 | 3.6 ± 0.1 | |
| Weight at birth (g) | 3441 ± 103 | 3358 ± 159 | 3295 ± 73 | 3281 ± 108 | |
| Head circumference at birth (cm) | 34.3 ± 0.3 | 33.7 ± 0.5 | 34.0 ± 0.3 | 33.3 ± 0.4 | |
| Gestational age at birth (weeks) | 38.7 ± 0.3 | 38.8 ± 0.7 | 39.2 ± 0.3 | 39.2 ± 0.4 | |
| Apgar score | 9.5 ± 0.3 | 9.3 ± 0.3 | 9.6 ± 0.4 | 9.9 ± 0.1 | |
| Birth order | 1.4 ± 0.1 | 1.5 ± 0.2 | 1.6 ± 0.2 | 1.3 ± 0.1 | |
| Mother's age at birth | 28.9 ± 0.6 | 28.4 ± 0.8 | 27.3 ± 0.9** | 31.4 ± 0.8 | $p<0.01$ |
| Father's age at birth | 30.9 ± 0.7 | 31.3 ± 0.9 | 28.9 ± 1.0* | 33.2 ± 1.3 | $p=0.01$ |
| | | | | | |
| GROUP II (age 7-9 years) | Autistic | Control | Autistic | Control | |
| N | 23 | 18 | 13 | 19 | |
| Mean age | 8.2 ± 0.1 | 8.4 ± 0.2 | 7.9 ± 0,2 | 8.4 ± 0.1 | |
| Weight at birth (g) | 3329 ± 183 | 3486 ± 151 | 3066 ± 183* | 3402 ± 108 | $p=0.02$ |
| Head circumference at birth (cm) | 33.9 ± 0.7 | 34.3 ± 0.5 | 33.2 ± 0.6 | 33.7 ± 0.4 | |
| Gestational age at birth (weeks) | 38.2 ± 0.7 | 38.8 ± 0.4 | 38.7 ± 0.6 | 39.4 ± 0.4 | |
| Apgar score | 8.7 ± 0.5 | 9.4 ± 0.2 | 9.0 ± 0.4 | 9.6 ± 0.2 | |
| Birth order | 1.4 ± 0.1 | 1.7 ± 0.2 | 2.3 ± 0.8* | 1.2 ± 0.1 | $p=0.03$ |
| Mother's age at birth | 27.3 ± 0.7 | 28.3 ± 1.0 | 28.5 ± 1.4 | 27.3 ± 0.9 | |
| Father's age at birth | 29.5 ± 0.8 | 30.0 ± 0.9 | 30.3 ± 1.3 | 28.7 ± 0.8 | |

Autistic and control study participants were divided into two age-groups, as indicated. Statistically significant differences between autistic and control groups are denoted by (*) and (**).

### Statistics

The STATISTICA software package for Windows (StatSoft, Tulsa, OK, USA) was used to analyze all data. Student's *t*-test was used when means of data from two groups were compared. *U*–Mann-Whitney test was used for comparisons of nonparametric data, McNemar's test with $\chi^2$ statistics was applied for categorical variables ('yes' or 'no'). For comparisons of mercury levels in hair 2-way ANOVA (disease x age) was utilized. Newman-Keuls test was used for individual post-hoc comparisons. Results with *p*-level less than 0.05 were considered significant. The results are presented as mean ± standard error of mean (SEM).

## RESULTS

### Demographics and birth-related measures

Altogether 91 autistic children and 75 age- and sex-matched healthy control children were enrolled into the study. The demographic data on participants is shown in Table I. There were no statistically significant age differences at the time of psychiatric examination and collection of specimens between the autistic and control groups. Most autistic and control children did not differ significantly with respect to their birth weights, except for a slightly lower weights of autistic girls from group II ($p$=0.02). Also head circumference at birth was not significantly different between autistic and control children, although in group I, both in males and females, there was a trend for slightly larger head size in autistics than in controls. This difference did not reach statistical significance ($p$=0.07). Likewise, Apgar scores were not statistically significantly different between autistic and control children, except that there appeared to be a tendency for slightly lower values for autistic children from group II. The only statistically significant difference in Apgar scores was between control males and females, with females having higher scores ($p$=0.04). The autistic and control groups did not vary significantly with respect to their

Table II

Comparison or clinical features between autistic and control children and between autistic male and females within each age-group

| Clinical Features | Males | | Females | | *p* (t-Student , U-M-W or $\lambda^2$) |
|---|---|---|---|---|---|
| GROUP I (age 3-4 years) | Autistic | Control | Autistic | Control | |
| Allergies (%) | 50 | 22.7 | 38.5 | 36.8 | |
| Number of vaccines till Year 2 | 24.5 ± 0.9 | 23.6 ± 0.7 | 24.6 ± 0.6 | 24.2 ± 0.6 | |
| Maximal number vac. at once | 5.0 ± 0.2 | 4.8 ± 0.1 | 4.8 ± 0.1 | 5.1 ± 0.2 | |
| Vaccine complications (%) | 38.5* | 4.5 | 15.4 | 5.3 | $p$=0.03 |
| Abnormal development (%) | 11.5 | 0 | 38** | 0 | $p$<0.01 |
| Regress (%) | 80.8 | | 81 | | |
| Hyperactivity (%) | 26.9 | | 34.6 | | |
| CARS total scores | 43.6 ± 1.3 | | 45.5 ± 1.2 | | |
| DSM IV A | 9.2 ± 0.3 | | 9.6 ± 0.2 | | |
| Autism-vaccine connection (%) | 19.2 | | 15.4 | | |

| Clinical Features | Males | | Females | | *p* (t-Student , U-M-W or $\lambda^2$) |
|---|---|---|---|---|---|
| GROUP II (age 7-9 years) | Autistic | Control | Autistic | Control | |
| Allergies (%) | 50 | 40 | 33.3 | 33.3 | |
| Number of vaccines till Year 2 | 22.3 ± 0.7 | 24.2 ± 0.7 | 20.2 ± 0.9** | 24.2 ± 0.5 | $p<0.001$ |
| Maximal number vac. at once | 4.6 ± 0.1 | 4.8 ± 0.1 | 4.3 ± 0.1* | 4.9 ± 0.1 | $p=0.03$ |
| Vaccine complications (%) | 16.7 | 10 | 26.7 | 9.2 | |
| Abnormal development (%) | 25 | 0 | 26.7 | 9.5 | |
| Regress (%) | 45.8 | 0 | 80 | 0 | |
| Hyperactivity (%) | 50 | | 40 | | |
| CARS total scores | 38.7 ± 1.2* | | 44.3 ± 1.8 | | $p=0.02$ |
| DSM IV A | 10.0 ± 0.3 | | 10.0 ± 0.3 | | |
| Autism-vaccine connection (%) | 12.5 | | 26.7 | | |

Autistic and control study participants were divided into two age-groups, as indicated. Statistically significant differences between autistic and control groups, or between autistic males and females are denoted by (*) and (**).

birth order, except again for the females from group II, who were of higher order (2.3) than controls (1.2; $p=0.03$). Autistic and healthy groups also did not diverge significantly with respect to parental age at child's birth, except for autistic girls from group I, who had slightly younger parents than the controls ($p\leq 0.01$).

**Clinical parameters**

Comparisons of major clinical features of autistic and control children are shown in Table II. There was a good correlation between CARS and DSM IV autism diagnostics ($r=0.74$). In the younger group of autistic children CARS and DSM IV scores were not notably dissimilar between males and females. However, in the older group of autistic children, the females appeared to be more impaired, as evidenced by their statistically significantly higher CARS scores than those in males ($p<0.02$). Generally, autistic and control children did not diverge significantly in the number of vaccinations received up to the 24$^{th}$ month of life, except for the autistic girls from the group II, who received fewer vaccinations ($p<0.001$) due to more frequent vaccine adverse events.

Autistic children from combined groups I and II experienced significantly more vaccine complications (20.4%) than controls (6.5%). This difference was statistically significant ($\chi 2=6.75$; $p=0.009$ (Table III). It was particularly pronounced in the males from group I, where 38.5% of autistics had adverse reactions to vaccines, while in the control group only 4.5% manifested such reactions ($p=0.03$, Table II). Vaccine complications reported by parents of autistic children included: high fever, prolonged crying, extended hypoactivity and hypotonicity, loss of contact, loss of facial mimicry, sleepiness, circling around, loss or ability to walk, point or talk, developmental regress, emergence of autistic behaviors. The vaccines most frequently associated with these adverse reactions, were: DTP, DTP-polio, DTP-Hib, DTP-polio-Hib, MMR, pneumococcal vaccine. A few adverse vaccination events reported by parents of control children included: skin reaction, crying and fever. Autistic patients also

diverged from controls in developmental characteristics, where 40.9% of autistics versus 3.9% of controls were reported to have abnormal development ($\chi 2=30.6$; $p<0.001$). Among autistic cohort developmental regress was noted in about 80%, except for the males from group II, where it was 46% (Table II). Comorbid hyperactivity was diagnosed in approximately 30% of the autistic children from group I, and in 45% of such children from group II. Among autistic boys the frequency of allergies (reported by parents) also appeared to be slightly higher than in controls, but the distinction was not statistically significant.

### Hair mercury content

Autistic and control children from both age-groups differed noticeably in the concentration of mercury in hair (Fig. 1). In group I, hair mercury levels were lower in autistic than in control children, but the situation was opposite in group II, where autistics had higher levels than controls ($p=0.01$). Consequently, there appeared to be opposing developmental trends between autistic and control children with respect to change of hair mercury levels. In autistics these levels increased with advancing age (from 3-4 to 7-9 years), whereas in controls – decreased.

## DISCUSSION

This study compared autistic and healthy control children of both sexes aged 3-4 and 7-9 years with respect to perinatal morphometric and clinical measures, abnormal development, vaccination history and mercury content in hair. The results point to statistically significant differences between autistic and control cohorts in three major categories. Autistic children had: 1) greater prevalence of abnormal development; 2) more frequent vaccine complications; 3) different concentrations of mercury in hair (younger autistics had lower levels, while older – higher levels than their age-matched controls).

For three out of four experimental (age-sex) comparison groups, the demographic and birth morphometric measures of autistic children were not statistically significantly different from the controls. Only the

Table III

Comparison of combined groups of autistic and control children

| | Autistic (M+F) Groups I + II | Controls (M+F) Groups I + II | *p* |
|---|---|---|---|
| Vaccine complications (%) | 20.4* | 6.5 | $p=0.009$ |
| Abnormal development (%) | 40.9* | 3.9 | $p<0.001$ |
| Caesarian or pathological birth (%) | 32 | 29 | NS |
| Epilepsy (%) | 5.5 | 1.2 | NS |
| Potential Rh conflict (%) | 8 | 12 | NS |
| Genetic load (%) | 12 | 5 | NS |

Nonparametric measures: Comparison of nonparametric measures between combined groups of autistic and control children. Information about epilepsy, potential genetic load and potential Rh conflict is based on parental interviews. M = males, F = females. Statistically significant differences are denoted by (*).

autistic girls from group II appeared at birth to be somewhat more disadvantaged, as they weighed less and were of a greater birth order than the controls. Greater impairment of this group was also evidenced by their higher CARS scores, when compared to autistic boys from the same age-group. Moreover, in group II, both autistic boys and girls had slightly lower Apgar scores than controls, but these differences did not reach statistical significance. Thus in the present study, obvious perinatal disadvantage did not appear to be a universal feature distinguishing the autistic from the control children, although a slight tendency for greater weakness at birth of some autistic children was noted. Even though this difference was not statistically significant for all experimental groups, its clinical significance for autism development cannot be entirely ruled out.

The most intriguing observation of this study is a significant difference in concentrations of hair mercury between autistic and control children, which was present in both age-groups, albeit with opposite developmental trends. In the autistic children, hair mercury levels were lower at a younger age and increased with development, whereas in the control children these levels were higher at a younger age and declined with development. In humans mercury content in hair is a biomarker of past exposure (Clarkson 1993, Gosselin et al. 2006), although hair appears to be a minor route of elimination of heavy metals from the body (Magos and Clarkson 2008). Typically hair mercury levels correlate with blood levels, but not necessarily with the burden to various tissues and the whole body (Nielsen et al. 1994). Studies with infant monkeys and rats showed that particularly organomercurials, which easily penetrate the blood brain-barrier and cell membranes, accumulate in the brain and other vital organs in much larger amounts than are present in blood, and they can stay in these organs for months or years (Burbacher et al. 2005, Olczak et al. 2009). The correlation or ratios of blood and hair mercury levels may be lessened in persons with inefficient cellular mechanisms of metals' elimination. Such a pattern was reported in autistic children (DeSoto and Hitlan 2007).

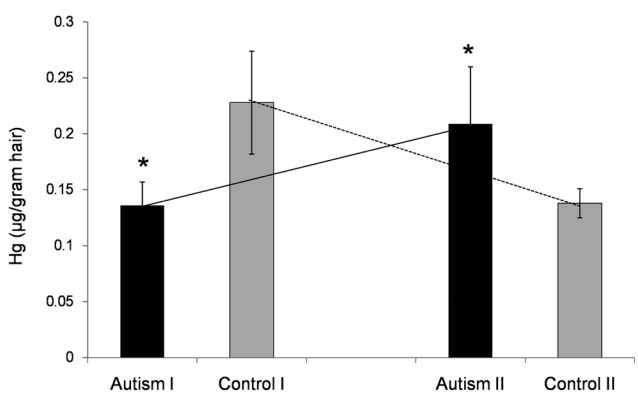


Fig. 1 Different levels of mercury in hair of autistic and healthy children from age groups I and II. The histogram shows mean values ± SEM. Statistically significant differences between autistic and control groups are denoted by (*), ($p$=0.01). Crossing lines point to divergent developmental trends of change in hair mercury levels between the autistic and control groups.

Our finding of lower concentrations of mercury in the hair of younger autistic children than in that of the healthy controls are qualitatively similar to the data of Holmes and colleagues (2003) and Adams and coauthors (2008) concerning first baby hair of American children. Lower hair levels of heavy metals, including mercury, in autistic children (1-6 years old) were also measured by Kern and others (2007). On the other hand, our results pertaining to older children – demonstrating higher mercury levels in the hair of autistic than control children – are comparable to the findings of Fido and Al-Saad (2005) in Kuwaiti boys (4-7 years old). It is important to stress, however, that the similarities with the latter study are only qualitative. While the concentrations of mercury in hair of control Kuwaiti boys (0,3 µg/g) were of the same order as in our study participants, the levels measured in autistic Kuwaiti children were 15 times higher (4,5 µg/g). These children must have been exposed to an extremely toxic environment, as they also had greatly increased levels of lead and uranium in their hair. Opposite developmental trends of hair mercury levels in autistic and healthy children may explain why in some studies, which used children of mixed ages, the difference in hair mercury levels between these cohorts was statistically insignificant (Kern et al. 2007, Ip et al. 2004). DeSoto and Hitlan (2007), who reanalyzed the dataset of Ip and coworkers (2004) that was originally analyzed with error (Wong 2007) found a significant correlation of autism diagnosis with higher levels of mercury in blood, but not in hair in Chinese children approximately 7 years old.

We did not scrutinize in detail the sources of mercury exposure in our study participants. It could be both prenatal and postnatal. Only one child – an autistic girl from the group II – had 5 amalgam fillings, which was highly unusual, as such procedures have been rarely used in children in Poland during the past 10 years. One of the greatest sources of prenatal mercury exposure, which increases vulnerability to autism, is the number and the age of maternal amalgam fillings (Drasch et al. 1994), because these fillings for years release significant amounts of mercury vapor, which is easily absorbed by the lung tissue into the body. Also, mercury level in breast milk is influenced by the number, size and age of maternal dental amalgams. Over time of amalgam exposure, mercury accumulates in body tissues (Mutter et al. 2007). Furthermore, dental treatments during pregnancy (cleaning, polishing, insertion or removal of amalgam fillings) markedly increase maternal and fetal exposure to mercury.

From our data, there appeared to be no significant difference in the numbers of amalgam fillings between mothers of autistic and healthy children. Fifty-five percent of mothers of healthy children and 58% of mothers of autistic children did not have any amalgams. Others had from 1 to 8, but there was no significant difference between these two groups. We have no data regarding the age and size of maternal amalgam fillings, nor dental treatments during pregnancy. Therefore a possible influence of dental amalgam exposure during pregnancy and body burden of the infants at the time of birth cannot be excluded. In the studies of Holmes and coauthors (2003) and Geier and coworkers (2009b), dental amalgams during pregnancy increased the risk for autism or the risk for a high severity of autism. Nonetheless, it seems rather unlikely that maternal amalgams would significantly influence mercury levels in hair of children 3-9 years old. The most significant source of mercury exposure in the studied population is from THIM-containing vaccines, the environment, including foodstuffs, and breast milk for the younger group. In this study, we have not examined the influence and duration of breast feeding of amalgam bearing mothers. Even though we did not scrutinize the diets of autistic and healthy children, they were not reported to be markedly different with respect to potential methylmercury sources, although some autistic children were on gluten, casein and sugar free diets as part of their therapy. None of the children have undergone chelation treatment. Evaluation of the types of vaccines received by autistic and control children also did not show significant differences, suggesting that both groups were probably exposed to comparable doses of mercury from vaccinations.

Distinct levels of hair mercury in autistic and control children from the same age groups may result either from dissimilar environmental exposure or differences in efficiency of elimination of this metal. While the first possibility cannot be entirely ruled out, the second appears more probable. Since older children receive fewer vaccinations than younger, hair mercury content in 7-9 years old would be expected to be lower than in the younger group. Such a pattern was indeed observed in the control, but not in the autistic children, where it was opposite. Our data seem consistent with the notion that young autistic children might be poor eliminators of heavy metals – hence showing lower mercury levels in the hair – but may retain greater amounts of mercury in their body tissues, including the brain (Holmes et al. 2003, Adams et al. 2007, 2008). At adrenarche their toxin elimination capacity may improve, as reflected by higher levels of mercury in hair of older autistic children.

Vertebrates have several mechanisms of elimination and detoxification of heavy metals. They include a system of sulfur containing molecules, such as sulfhydryl- aminoacids and peptides – cysteine and reduced glutathione – as well as sulfates (Clarkson 1993, Bernard et al. 2001). Glutathione, synthesized by all mammalian cells is believed to serve as a primary heavy metal detoxifying molecule, which is excreted in bile as glutathione-metal complexes (Refsvik and Norseth 1975, Ballatori and Clarkson 1985). These sulfur-compounds are synthesized in various tissues, predominantly in the liver via the methionine transmethylation and transsulfuration metabolic pathways (Clarkson 1993, James et al. 2005). The mechanisms of mercury binding by cysteine and glutathione and its detoxification are complex, regulated by sex, age, genetic factors, and diets (milk diet decreases mercury excretion) (Rowland et al. 1984, Thomas et al. 1986, Clarkson 1993). Other heavy metal detoxifying molecules are cysteine rich proteins, metallothioneins (Piotrowski et al. 1974, West et al. 2008), the expression of which changes during postnatal development reaching adult levels in prepubertal age (Waalkes and

Klassen 1984). These factors may explain lower rates of heavy metals' excretion by suckling animals than by adults (Doherty et al. 1977, Ballatori and Clarkson 1982, Lok 1983), as well as our finding of apparently improved mercury elimination by older autistic children, as reflected by higher mercury levels in their hair.

Several studies reported deficiencies in autistic children metabolism of sulfur compounds, lower plasma concentrations of endogenous metabolites of transmethylation and transsulfuration such as methionine, S-adenosylmethionine, cysteine and reduced glutathione, but increased levels of oxidized glutathione and S-adenosylhomocysteine (Alberti et al. 1999, Kidd 2002, James et al. 2006, Geier et al. 2009). Some of these problems may ensue from the presence of susceptibility alleles, other may result from toxic effects of mercurials *per se* (James et al. 2005, 2006). Metabolic consequences of such defects include reduced detoxification of heavy metals, hence their increased toxicity, impaired methylation and redox homeostasis, and increased oxidative stress (Kern and Jones 2004, Zoroglu et al. 2004, James et al. 2006, Geier et al. 2009a), which adversely influence brain development and CNS functions.

Lower levels of mercury in hair of young autistic children may suggest reduced ability to excrete metals, resulting in high burden of mercury and increased vulnerability to its neurotoxic effects. This might ensue from genetic factors or from certain comorbid pathologies. For example, Prandota (2009) recently proposed that autism spectrum disorders may be linked to cerebral toxoplasmosis, which results in hypercytokinemia and makes infants more vulnerable to environmental insults, including mercurials and vaccinations. This intriguing hypothesis requires experimental verification. A direct evidence for greater prenatal and postnatal mercury burden in autistic children comes from research showing higher levels of this metal in baby teeth of autistic children than in controls (Adams et al. 2007) and from a study documenting increased urinary excretion of this metal by autistic children after treatment with chelating agent (Bradstreet et al. 2003). Also augmented concentrations of atypical urinary porphyrins (specific for mercury exposure) in autistic children suggest heavy mercury burden (Woods et al. 2005, Nataf et al. 2006, Geier et al. 2009a).

In our study participants, the source of mercury exposure is probably mixed. Nonetheless, because THIM-containing pediatric vaccines are still used in Poland (although they were abandoned by most developed countries due to toxicity concerns), and the autistic children manifested higher incidences of serious adverse reactions to vaccinations, an iatrogenic effect of THIM in this population is possible. Such effect was documented in American boys immunized at infancy with THIM-containing Hep-B vaccines, who were 9 times more likely to suffer from learning disabilities than those who did not receive these vaccines (Gallagher and Goodman 2008). Furthermore, vaccination of infant boys with Hep-B vaccines tripled their risk of developing autism, when compared to unvaccinated children (Gallagher and Goodman 2009). The neurotoxic effect of THIM-containing Hep-B vaccine was recently confirmed in newborn monkeys, which after receiving its single dose manifested delayed acquisition of vital neonatal reflexes (Hewitson et al. 2009). In view of the growing body of clinical and preclinical evidence of strong toxicity of all forms of mercury in developing organisms, the removal of THIM from all vaccines given to children and pregnant women is urgently required.

### Strengths and limitations

The major strength of this study is its controlled nature, uniformed selection of study participants from the country, which still uses THIM in pediatric in vaccines, utilization of semistructured parental interview and child diagnosis conducted by the same team of experienced professionals, comprehensive inspection of patients' medical records, and use of age- and sex- differentiated groups. The weaknesses include inability to assess more accurately the sources of mercury exposure and non-uniform selection of study participants: controls were from one geographic region, while autistic patients were from more diverse regions of Poland. (Nonetheless, only one autistic child was from heavy industrial region, but his level of hair mercury was not markedly different from the rest of his age group).

## CONCLUSION

Autistic and healthy children differ in prevalence of abnormal development, frequency of adverse reactions to vaccinations and concentrations of mercury in hair, which change with development. The data indirectly imply vaccinations and mercurials as potential factors in autism pathogenesis.

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