



# EXPOSURE OF CHILDREN WITH AUTISM SPECTRUM DISORDERS TO MERCURY AND POLYCYCLIC AROMATIC HYDROCARBONS

## NARAŻENIE DZIECI Z ZABURZENIAMI ZE SPEKTRUM AUTYZMU NA RTĘĆ I WIELOPIERŚCIENIOWE WĘGLOWODORY AROMATYCZNE

Śławomir Waligóra<sup>1</sup>, Aleksandra Waligóra<sup>1</sup>,  
Aleksandra Damasiewicz-Bodzek<sup>1</sup>, Piotr Gorczyca<sup>2</sup>,  
Krystyna Tyrpień-Golder<sup>1</sup>

Correspondence to/  
Adres do korespondencji:

Aleksandra Waligóra  
Department of Chemistry  
School of Medicine  
with the Division of Dentistry in Zabrze  
Medical University of Silesia in Katowice  
19 Jordana St.  
41-808 Zabrze, Poland  
e-mail: awaligora@sum.edu.pl

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<sup>1</sup>Department of Chemistry, School of Medicine with the Division of Dentistry,  
Zabrze, Medical University of Silesia, Katowice, Poland

<sup>2</sup>Department of Psychiatry, School of Medicine with the Division of Dentistry,  
Zabrze, Medical University of Silesia, Katowice, Poland

<sup>1</sup>*Katedra i Zakład Chemii, Wydział Nauk Medycznych w Zabrze, Śląski Uniwersytet Medyczny w Katowicach, Polska*

<sup>2</sup>*Katedra Psychiatrii, Wydział Nauk Medycznych w Zabrze, Śląski Uniwersytet Medyczny w Katowicach, Polska*

### Abstract

**Purpose:** Autism is described as a developmental disorder with numerous impairments in the functioning of the central nervous system. Contact with poisonous chemicals present in the environment and food is a major factor which interferes with attempts to minimize the symptoms of this disorder. Thus, the effectiveness of the use of natural mechanisms for the elimination of toxins from organisms in the case of people with autism spectrum disorders (ASD) may not be sufficient. As part of the evaluation of exposure to polycyclic aromatic hydrocarbons (PAHs) and mercury compounds, the determination of the presence of 1-hydroxypyrene (1-OHP) in urine samples and total mercury in the hair and urine of children with ASD and a control group originating from the Upper Silesian region was carried out.

**Methods:** Thermal decomposition of mercury compounds, amalgamation and determination of its total concentration by atomic absorption spectrometry (AAS) was carried out. 1-OHP was determined by high-performance liquid chromatography (HPLC) with a fluorescence detector after prior sample preparation by solid phase extraction (SPE).

**Results:** The median mercury concentration was 0.142 µg/g for hair samples and 0.165 µg/g creatinine for urine samples in the group of children with ASD, while for the control group 0.102 µg/g of hair and 0.140 µg/g creatinine were obtained respectively. The median concentration of 1-OHP in urine for the ASD group was 173.769 ng/g creatinine, while for the control group it was 124.157 ng/g creatinine.

**Conclusions:** No significant difference in the concentrations of both total mercury and 1-OHP between the test and control groups were observed. There was no increased exposure of children with ASD to environmental factors compared to healthy children.

**Key words:** mercury, 1-hydroxypyrene, polycyclic aromatic hydrocarbons, urine, autism spectrum disorders (ASD).

### Streszczenie

**Cel:** Autyzm jest opisywany jako zaburzenie rozwojowe o licznych zakłóceniach w funkcjonowaniu ośrodkowego układu nerwowego. Kontakt z trującymi chemikaliami obecnymi w środowisku i pożywieniu to główny czynnik, który uniemożliwia minimalizowanie objawów tego zaburzenia. Tak więc skuteczność naturalnych mechanizmów eliminacji toksyn z organizmu u osób z zaburzeniami ze spektrum autyzmu (ASD) może nie być wystarczająco skuteczna. W ramach oceny narażenia na wielopierścieniowe węglowodory aromatyczne (WWA) i związki rtęci zostało przeprowadzone oznaczenie 1-hydroksypirenu (1-OHP)

w próbkach moczu i całkowitej zawartości rtęci we włosach i moczu dzieci z ASD i grupy kontrolnej pochodzących z regionu Górnego Śląska.

**Metody:** Przeprowadzono termiczny rozkład związków rtęci, amalgamację i oznaczenie całkowitego stężenia metodą spektrometrii absorpcji atomowej (AAS). 1-Hydroksypiren oznaczono metodą wysokosprawnej chromatografii cieczowej (HPLC) z detektorem fluorescencyjnym po uprzednim przygotowaniu próbki metodą ekstrakcji w fazie stałej (SPE).

**Wyniki:** Mediana koncentracji rtęci wynosiła 0,142 µg/g dla próbek włosów i 0,165 µg/g kreatyniny dla próbek moczu w grupie dzieci z ASD, podczas gdy dla grupy kontrolnej uzyskano, odpowiednio, 0,102 µg/g dla próbek włosów i 0,140 µg/g dla próbek moczu. Mediana stężenia 1-OHP w moczu dla grupy ASD wynosiła 173,769 ng/g kreatyniny, podczas gdy dla grupy kontrolnej 124,157 ng/g kreatyniny.

**Wnioski:** Nie zanotowano znaczącej różnicy w stężeniach zarówno rtęci całkowitej, jak i 1-OHP pomiędzy grupą badaną i kontrolną. Nie stwierdzono zwiększonego narażenia dzieci z ASD na czynniki środowiskowe w porównaniu z dziećmi z grupy kontrolnej.

**Słowa kluczowe:** rtęć, 1-hydroksypiren, wielopierścieniowe węglowodory aromatyczne, mocz, zaburzenia ze spektrum autyzmu (ASD).

## INTRODUCTION

Autism is currently regarded as a complex developmental disorder, the definition of which is constantly being expanded and verified. Autism spectrum disorder (ASD), a relatively new term in the literature, was introduced in 2013 by the American Psychiatric Association in the diagnostic and statistical manual of psychiatric disorders (DSM-V). The term, which is not the same as autism, also includes Asperger's syndrome and pervasive developmental disorders not otherwise specified (PDD-NOS). The diagnosis of ASD, according to DSM-V, is based on the identification of permanent deficits in social communication and social interactions as well as limited and repetitive patterns of behaviour. In addition, symptoms should appear in an early developmental period and the clinical picture cannot be explained by other mental disorders [1]. In the majority of European countries, including Poland, the International Statistical Classification of Diseases and Related Health Problems (ICD-10), which was developed by the World Health Organization (WHO), is recognised. The ICD-10 classification defines a group of disorders referred to as overall developmental disorders, and the children's autism attributed to it, as a complex disorder in the context of social interactions, communication, and behaviour, manifested before the age three [2].

In 2017, a meta-analysis was published which examined the relationship between prenatal, perinatal and postnatal (neonatal) factors and the risk of autism. Many lines of evidence suggest that changes in the brain development occur long before the appearance of diagnosable symptoms. The etiology of autism spectrum disorders has a strong genetic basis. Studies of autistic twins have estimated the contribution of genes predisposing children to the development of autism disorders. Depending on the analysis of the heritability index, such studies estimate that the proportion of phenotypic variation caused

by genetic factors can be as high as 83% (analysis of over thirty-seven thousand pairs of twins, over two million pairs of siblings) [3]. Ronald and Hoekstra had reviewed over 30 studies involving mono- (MZ) and dizygotic (DZ) twins, on the basis of which they reported high and comparable heritability of symptoms characteristic for ASD. In the case of studies in which autism was defined on the basis of a narrower range of symptoms, the median value of the concordance for MZ and DZ was 76% and 0%, respectively. However, for studies in which a group of twins was classified as ASD with broader criteria, the median of concordance was 88% and 31% for MZ and DZ [4]. Colvert *et al.* estimated ASD heritability at 56-96%, reporting a significantly higher correlation for MZ (0.77-0.99) than for DZ (0.22-0.65) [5]. In contrast, Hallmayer *et al.*, who studied 192 pairs of twins, reported only a moderate influence of genetic factors (about 38%), but with a significant influence of environmental factors on the etiology of ASD (about 58%) [6]. These data show that the estimates of genetic inheritance differ, but they nevertheless reinforce the hypothesis that the development of ASD is also influenced by environmental factors. Therefore, there is a notion that ASD has a multifactorial etiology, in which increasing attention is given to the role of the environment.

In addition to studies confirming the existence of a relationship between environmental factors in people genetically predisposed to autism disorders and the development of ASD, studies focusing on patients' exposure to harmful doses of exogenous substances are also undoubtedly of great importance. The occurrence of chronic oxidative stress [7, 8], impairment of essential metabolic pathways that produce antioxidant enzymes [9], and significantly elevated expression of the metallothionein, used for the detoxification of heavy metals [10] provide the basis for the hypothesis that a high proportion of people with ASD have been affected by xenobiotics. An ineffective system of detoxification may predispose them to the occurrence of biological anomalies with a neuro-

chemical and immune background, inducing in them behavioural traits characteristic of ASD. Particular attention is paid in this regard to certain heavy metals, such as mercury. It has been reported that the concentration of mercury in the blood can positively correlate with the presence of antibodies to myelin basic protein (anti-MBP) [11-13]. Fel *et al.* [14], Hodgson *et al.* [15], Al-Ayadhi [16] and Blaurock-Bush *et al.* [17], who obtained significantly higher mean mercury concentrations in the hair of children in an autistic group than to a control group. Moreover, studies have shown that the concentration of mercury in hair samples [18] and in red blood cells [19, 20] may positively correlate with the degree of intensification of autism symptoms. Mercury, then, has repeatedly been proposed as a cause of autism. A review of the literature by Kern [21] reveals that a large majority of the work (approx. 74%) under consideration took into account the role of mercury as a risk factor for ASD. Mohamed *et al.* [22] noted a statistically significant relationship between maternal intake of fish during pregnancy and the content of elemental mercury in the hair of autistic people. So far, no evidence has been found to suggest prenatal exposure to maternal mercury is the cause of autism. However, researchers are increasingly focused on looking for a connection between mercury exposure and the manifestation of extreme features characteristic of deficits in a specific social skill [23].

Next to the heavy metals, polycyclic aromatic hydrocarbons (PAHs) are the most widespread group of environmental pollutants. The presence of 1-hydroxypyrene (1-OHP) is considered to be an indicator of the exposure of humans to PAHs. Demographic factors and diet are the main sources of exposure of young people to PAH. In 2016, WHO published a report containing a list of 50 cities within the European Union with exceeded the PM 2.5 dust concentration limits in the air. The list includes 33 Polish cities, a large number of them located in the highly urbanized region of Silesia. PAHs occur in the gaseous phase and are adsorbed on suspended particulate matter (PM), from where they later penetrate into waters, soils, and sediments. The concentrations of some PAHs (e.g. Benzo[a]pyrene) associated with exposure to PM 2.5 of dust measured on an annual basis in some cities in the region are comparable to those measured in the large Asian agglomerations [24]. It is suspected that high levels of air pollution may increase the risk of autism spectrum disorders in people with genotype MET rs1858830 CC (receptor tyrosine kinase gene variant rs1858830), on the basis of gene-environment interactions [25]. The data show that exposure to PAH, expressed in the concentration of 1-OHP in the urine, has a positive effect on the concentration of 8-hydroxy-2-deoxyguanosine, widely regarded as a biomarker of oxidative stress [26]. PAHs have a negative impact on cognitive development in children who were exposed to them during the prenatal period [27].

Moreover, studies indicate an increased risk of autism spectrum disorders during pregnancy and in the first year of life of children living in areas with high levels of local traffic-related air pollution [28].

The aim of the study was to determine the presence of 1-OHP as a biomarker of PAHs in urine samples and total mercury in the hair and urine of children with ASD and a control group from Silesia in Poland. These determinants allow for the comparison of the two groups in terms of the loading of these factors and checking of whether there is a clear difference between them that could result from an existing neurodevelopmental disorder, which is ASD.

## METHODS

The study protocol was approved by the local Bioethics Commission by resolution no. KNW/0022/KB1/155/IV/08/11/12/14 of 17/06/2014.

### Inclusion criteria

The study involved children under 15 years old of both genders from the Silesian agglomeration in Poland who had been diagnosed with autism spectrum disorders by a psychiatrist. The control group consisted of children without ASD of similar age and of both genders from the same geographic area.

### Survey and statistics

Before the biological material was taken, an original questionnaire was completed with the parents of autistic children. Based on this, socio-demographic information about children with ASD and their caregivers was obtained. Information on children's nutrition and the presence of selected environmental pollutants was also obtained.

The results were compiled using basic parameters of descriptive statistics. Conformity with normal distribution was checked by the Shapiro-Wilk test. Independent data between groups of children with autism and the control group were compared using the *U* Mann-Whitney test (data distribution was inconsistent with normal distribution). The significance level  $p < 0.05$  was assumed to be statistically significant.

### Material

Hair samples were collected from 20 children (aged 2-15 years) with diagnosed autism spectrum disorders ( $N_A$ ). The control group ( $N_k$ ) consisted of 18 healthy children under the age of 15. The hairs (about 1 g) were cut from the occipital part of the head. Morning urine samples were collected for the determination of the presence of 1-OHP ( $N_A = 13$ ,  $N_k = 19$ ) and mercury ( $N_A = 16$ ,  $N_k = 20$ ) and stored at  $-20^\circ\text{C}$  in polypropylene test tubes. The daytime

collection of urine from children is problematic, so it was decided that the urine samples would be collected once a day. Siwińska *et al.* [29] suggest introducing a reference parameter to avoid the variation resulting from inconstant dilution of the samples. 1-OHP and total mercury concentrations were referenced to the unit of creatinine content.

## Mercury

The method was based on the thermal decomposition of mercury compounds – amalgamation and determination of total mercury in the biological material by atomic spectrometry at a wavelength of 253.7 nm, using a mercury analyzer DMA-80 from Milestone. Measurement of mercury in urine and hair samples was carried out at the Nofer Institute of Occupational Medicine in Łódź (Poland).

## 1-Hydroxypyrene

Urine samples (10 ml) were adjusted to pH = 5.5 by adding 0.1 M HCl. Then we added 10 mg of sodium azide, 40 µl of β-glucuronidase/arylsulfatase (Helix pomatia, Calbiochem), 2 ml of 1 M acetate buffer and internal standard (1.8 ng/µl) – 1-pyrenebutyric acid (Fluka). The samples were incubated for 16 hours at 37°C and then subjected to extraction, which was carried out using C18 columns (500 mg/6 ml Bakerbond SPE). Columns were conditioned with 5 ml of methanol and 10 ml of distilled water. After applying the sample, columns were washed with 10 ml of distilled water and dried. 1-OHP was eluted with 5 ml of methanol: acetone (1 : 1). The collected fractions were evaporated to dryness in a stream of nitrogen and then dissolved in 400 µl of methanol.

Concentrations of 1-OHP in urine samples were determined using a high-performance liquid chromatography HPLC technique with a fluorescence detector (Dionex Ultimate 3000 HPLC System with RF2000 FD). The calibration graph for 1-OHP in methanol was made in the concentration range 0.00625-0.1 ng/µl. Isolated fractions were separated on a Luna C18 column (5 µm, 100Å, Phenomenex) using a gradient; mobile phase: 3 min 80% eluent A (40% methanol in water) and 20% eluent B (100% methanol); 0-15 min linear increase to 75% solution B, 18-25 min linear growth of eluent B to 100%; flow rate: 0.8 ml/min. The excitation wavelength was 242 nm and the emission wavelength was set at 389 nm. Calibration of 1-OHP was carried out in the range of 0.005-0.16 ng/ml [30].

## RESULTS

### Characteristics of the population studied

The study group comprised 80% of boys and 20% of girls. Children were divided into three age ranges: 0-5 years (4%), 6-10 years (48%) and 11-15 years (44%). All examined children lived in Upper Silesia. The smallest por-

portion of respondents lived in rural areas and small towns (under 10,000 residents), while the largest group came from cities with a population over 100,000 (Figure I).

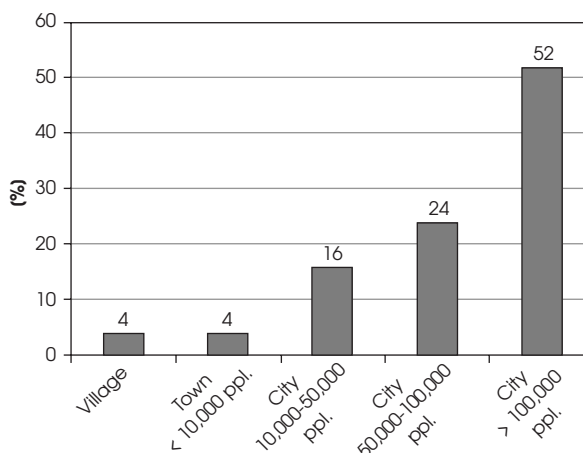


Figure I. Percentage distribution of the autism spectrum disorder (ASD) group on the basis of place of residence

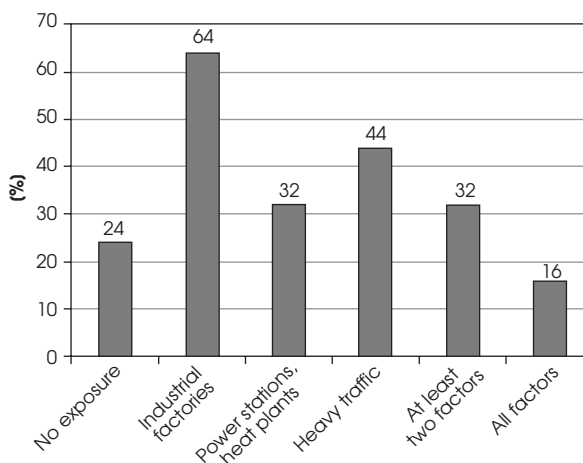


Figure II. Percentage distribution of the autism spectrum disorder (ASD) group divided into areas with selected environmental hazards

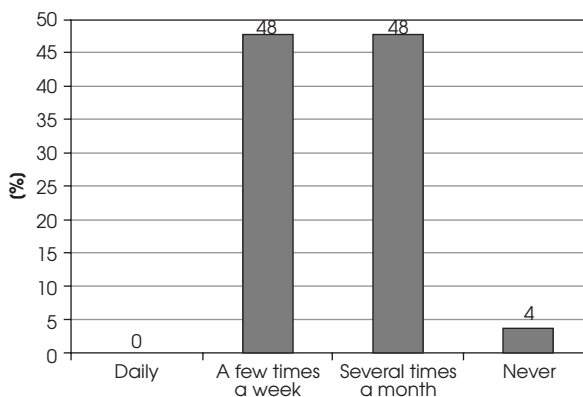
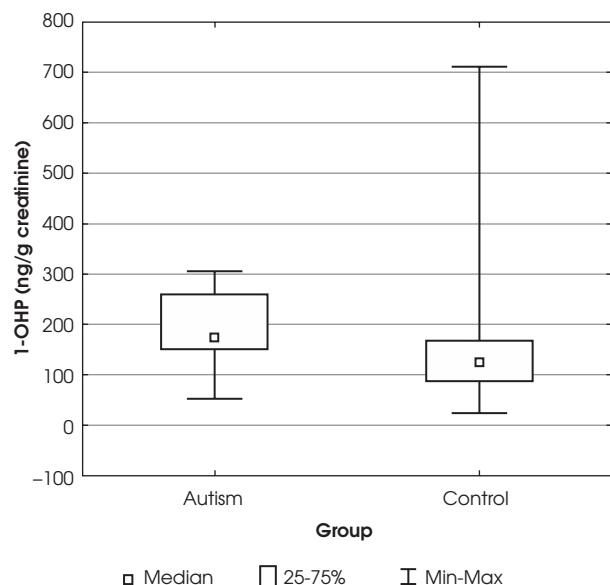


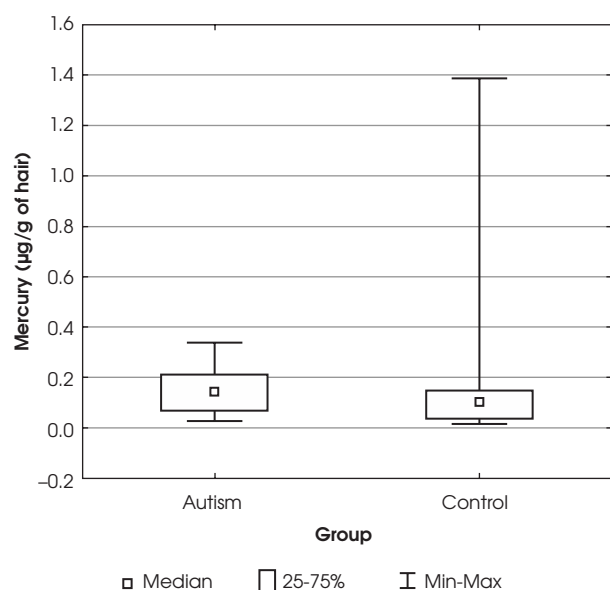
Figure III. The frequency of fish consumption by children with autism spectrum disorder (ASD)

**Table 1.** Descriptive statistics of obtained results

Study	Group	Group size	Mean	Median	Maximum	Minimum	Standard deviation
1-OHP (ng/g creatinine)	Autism	13	189.8245	173.7690	305.9480	52.1405	84.8880
	Control	19	180.1866	124.1571	711.1031	22.2460	166.3403
Mercury in hair (µg/g of hair)	Autism	20	0.1521	0.1420	0.3370	0.0330	0.0914
	Control	18	0.2321	0.1015	1.3880	0.0180	0.3533
Mercury in urine (µg/g creatinine)	Autism	16	0.2619	0.1650	0.8300	0.0800	0.2058
	Control	20	0.2075	0.1400	0.8000	0.0200	0.2120



**Figure IV.** Comparison of urinary 1-OHP concentration between autism spectrum disorder (ASD) and control group



**Figure V.** Comparison of mercury concentration between autism spectrum disorder (ASD) and control group in hair samples

The survey also showed a high percentage of families living in areas near large industrial plants, high-level of road traffic or close to combined heat and power plants (CHP). The place of residence was often in a neighbourhood with as many as two of the three aforementioned environmental pollution factors, which could be a significant source of PAHs in these locations (Figure II).

The questionnaire included the dietary habits of children in the study group. The high frequency of the consumption of fish, which is an important source of mercury, was noteworthy. Research shows that fish are a common component of the diet of these children (Figure III).

The following table presents descriptive statistics on determinations of the presence of 1-OHP and mercury in biological material for two groups: the ASD and control groups (Table 1).

### Exposure to polycyclic aromatic hydrocarbons of children with autism spectrum disorder

The average 1-OHP concentration was 189.82 ng/g creatinine (median 173.77 ng/g creatinine) in the ASD group, while for the control group it was 180.19 ng/g creatinine (median 124.16 ng/g creatinine). There was no significant difference ( $p = 0.179, p > 0.05$ ) between the test group and the control group in the 1-OHP concentration. Figure IV shows the differences in 1-OHP concentrations in urine for both groups.

### Exposure to mercury of children with autism spectrum disorder

The average concentration of mercury in hair was 0.152 µg/g (median 0.142 µg/g of hair) in the ASD group, while for the control group it was 0.232 µg/g (median 0.102 µg/g of hair). The analysis showed no statistically significant difference ( $p = 0.306, p > 0.05$ ) of mercury concentrations in the hair between the test group and the control group (Figure V).

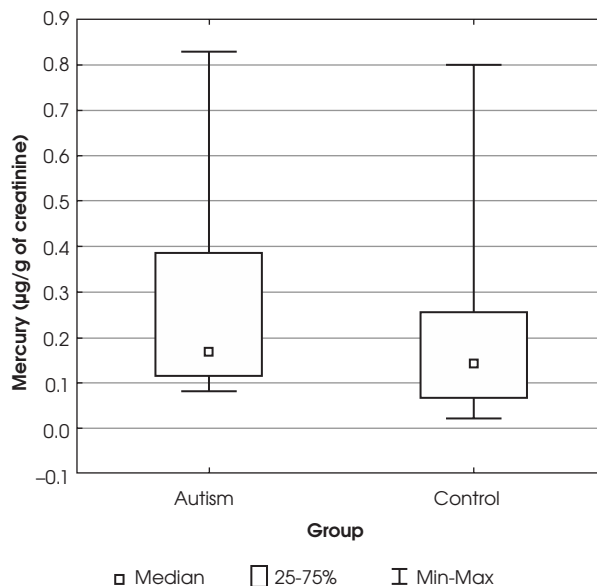
The mean concentration of mercury in the urine for test group was 0.262 µg/g creatinine (median 0.165 µg/g creatinine), while for the control group it was 0.208 µg/g creatinine (median 0.140 µg/g creatinine). Figure VI presents a graph of mercury concentrations in urine samples for both groups. The analysis also showed no statistical-

ly significant difference ( $p = 0.143$ ;  $p > 0.05$ ) of mercury concentration in the urine between groups.

## DISCUSSION

The safe limit of 1-OHP in the urine, above which genotoxic effects may appear, has not yet been established. 1-OHP is most often studied in the context of the assessment of the occupational exposure limits to dangerous substances such as PAHs. The approximate values of a 1-OHP concentration in the urine of employees, equivalent to the permissible total concentration of PAHs in the air at a given workplace (OEL – occupational exposure limit); these differ from one another depending on the sector of industry [30]. Studies with children from one of the most contaminated areas in Europe – the Silesian agglomeration – allow us to initially estimate the levels of 1-OHP in the urine of people not occupationally exposed to PAHs. The median concentration of 1-OHP for children from Sosnowiec was  $0.65 \mu\text{mol/mol}$  creatinine ( $0.65 \mu\text{g/g}$  creatinine for boys) and  $0.52 \mu\text{mol/mol}$  creatinine ( $0.52 \mu\text{g/g}$  creatinine for girls) [29]. Jongeneelen estimated exposure to PAHs for a group of children from various districts of Bytom was  $0.3\text{--}0.8 \mu\text{mol/mol}$  of creatinine ( $0.3\text{--}0.8 \mu\text{g/g}$  creatinine for both genders) [32]. In comparison to the above results, 1-OHP concentrations for children with ASD and healthy subjects were at a similar level. Thus, there was no more excessive exposure of children with ASD to such concentrations than those in the control group.

According to the Methylmercury-Environmental Health Criteria 101 report issued under the International Program for Chemical Safety (IPCS), the human population is mainly exposed to mercury through diet. The foods most abundant in mercury are fish and fish products. The report estimates that the daily intake of methylmercury is about  $2.4 \mu\text{g}$ , and the total daily dose of all forms of mercury is about  $6.8 \mu\text{g}$  [33]. According to previous studies, the concentration of mercury in the hair, which is mainly in the form of methylmercury, is usually less than  $1 \mu\text{g/g}$  of hair for people with low fish intake and does not exceed  $30 \mu\text{g/g}$  of hair for people who eat fish regularly (up to several times per week). Hair mercury can also come from exogenous sources. Mercury compounds can come from the air and can be adsorbed on the hair surface, depending on the form of occurrence, or embedded in the hair structure in various ways. Cleaning hair samples may not be advisable, because this process removes exogenous mercury and the estimation of the total amount of exogenous mercury becomes problematic [34]. The mean of total mercury concentration for a population with unknown occupational exposure to mercury and no exposure to mercury from fish consumption has been estimated to be less than  $10 \mu\text{g/kg}$  of urine [33]. The median of total mercury concentration in urine for samples from the Polish



**Figure VI.** Comparison of mercury concentration in autism spectrum disorder (ASD) and control group in urine samples

population in groups divided by gender did not exceed  $0.5 \mu\text{g/g}$  creatinine in any of the groups [35]. For comparison, the German Commission on Human Biological Monitoring has recommended a value of urinary mercury concentration equal to  $5 \mu\text{g/g}$  creatinine, below which there is no risk of toxic effects of poisoning, and a value of  $20 \mu\text{g/g}$  of creatinine, above which that risk is possible [36]. In order to estimate the differences in concentrations of this element between persons with ASD and healthy people, Saghazadeh *et al.* [37] performed a meta-analysis including 31 studies on the determination of the presence of mercury in hair and urine. A meta-analysis of 1092 patients with ASD and 973 controls did not confirm a significant difference in mercury concentrations in the hair between the groups. However, the inclusion of demographic criteria made it possible to show that significantly higher levels of mercury are found in the hair of ASD patients living in developing countries.

Differences of Hg concentration in the urine between the ASD (a total 198 patients) and control group (a total 258 patients) was also not statistically significant.

## CONCLUSIONS

In conclusion, no increased exposure of children with ASD to mercury in comparison with healthy children was found. Simultaneously, there was no increased risk of exposure to mercury in any of the groups. Additionally, in our study, differences in the concentration of PAHs were also not statistically significant. This might mean that the efficiency of elimination processes of mercury and PAHs is similar in both groups.

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### Conflict of interest/Konflikt interesu

Absent./Nie występuje.

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