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Regular paper

Determination of modified nucleosides in the urine of children with autism spectrum disorder

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Metabolic disorders and nutritional deficiencies in ASD children may be identified by the determination of urinary-modified compounds. In this study, levels of selected seven modified compounds: O-methylguanosine, 7-methylguanosine, 1-methyladenosine, 1-methylguanine, 7-methylguanine, 3-methyladenine, and 8-hydroxy-2`-deoxyguanosine in the group of 143 ASD children and 68 neurotypical controls were analyzed. An ancillary aim was to verify if the reported levels differed depending on the pathogenetic scoring of ASD (mild deficit, moderate deficit, severe deficit). Elevated O-methylguanosine and 7-methylguanosine levels and significantly lower levels of 3-methyladenine, 1-methylguanine, 1-methyladenosine, 7-methylguanine, and 8-hydroxy-'2'-deoxyguanosine were observed in ASD children compared to controls. O-methylguanosine levels were elevated in the mild and moderate groups, while the levels of 1-methylguanine, 1-methyladenosine, 7-methylguanine, and 8-hydroxy-'2'-deoxyguanosine in the same groups were lower than in neurotypical controls. The reported evidence shows that modified nucleosides/bases can play a potential role in the pathophysiology of ASD and that each nucleoside/base shows a unique pattern depending on the degree of the deficit.

Keywords: autism, methylation, modified nucleosides, bases

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Abbreviations: ADA, adenosine deaminase; ADSL, adenylosuccinate lyase; ANOVA, analysis of variance; ASD, autism spectrum disorder; ATP, adenosine triphosphate; AUC, area under the curve; GTP, guanosine triphosphate; HPRT, hypoxanthine-guanine phosphoribosyl transferase; LC-MS/MS, liquid chromatography coupled to mass spectrometry; NAPDD, nucleotidase-associated pervasive developmental disorder; PRPS, phosphoribosyl-pyrophosphate synthetase; ROC curves, receiver operating characteristic curves; ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; S.D., standard deviation

INTRODUCTION

Autism spectrum disorder (ASD) is a complex, pervasive developmental disorder with a neurological- etiopathogenetic basis, mainly occurring in childhood (Mughal & Saadabadi, 2020). It appears primarily as a qualitatively altered behaviour in social interaction, verbal and nonverbal communication. ASD covers a wide range of disorders, from mild to severe forms. "The Diagnostic and Statistical Manual of Mental Disorders, 5th Revision (DSM-5)" defines ASD as a neurodevelopmental disorder in which there are qualitative disorders in the field of social contacts, qualitative disorders in communication, which manifest themselves as stereotypical patterns of behaviour, interests, and life itself (American Psychiatric Association, 2013). ASD affects more than 1% (from 1 in 88 to 1 in 68) of children worldwide (male to female ratio of approximately 4:1) (Liu *et al.*, 2019). Generally, ASD is diagnosed based on assessing behavioural symptoms, including a questionnaire and a psychologist evaluation. Due to the early onset of symptoms (in the first two years of life) and their diversity, the diagnosis of ASD is difficult and often unreliable, especially at an earlier stage of development (Anwar *et al.*, 2018). Several studies indicate that there are metabolic disorders associated with ASD that can be assessed by identifying and determining characteristic biomarkers (Emanuele *et al.*, 2010; Kałużna-Czaplińska *et al.*, 2014; Żurawcz & Kałużna-Czaplińska, 2015; Kałużna-Czaplińska *et al.*, 2017; Kałużna-Czaplińska *et al.*, 2018; Adams *et al.*, 2019; Glinton & Elsea, 2019; Bitar *et al.*, 2020).

An accumulation of evidence suggests that environment-gene interactions may be critically important for the onset of ASD (Imamura *et al.*, 2020). The genetic endowment appears crucial to highlight both the etiopathogenesis and the development of ASD. However, different environmental impacts could play an important role in ASD onset and progress (Costa *et al.*, 2017; Ng *et al.*, 2017; Costa *et al.*, 2020; Ongono *et al.*, 2020; Radke *et al.*, 2020). Many pollutants are xenobiotics able to trigger the usual oxidative stress response of the individual. Under normal physiological conditions, the formation of free radicals and their neutralization is in a dynamic equilibrium. However, excessive production of reactive oxygen species and reduced antioxidant capacity, however, can lead to an imbalanced oxidative stress response, which

may contribute to the pathophysiology of ASD in predisposed individuals (Lambeth, 2007; Melnyk *et al.*, 2012; Ranjbar *et al.*, 2014; Kałużna-Czaplińska & Jóźwik-Pruska, 2016; Bobrowska-Korczak *et al.*, 2019; Osredkar *et al.*, 2019, Gątarek *et al.*, 2020). Nevertheless, excessive production of reactive oxygen species (ROS), particularly due to the imbalance between oxidants and antioxidants, can be toxic to neurons triggering DNA methylation (Wong *et al.*, 2019; Dreser, 2020; Sedley, 2020), which in turn means correlated damage to multiple further tissues (Ranjbar *et al.*, 2014). Several studies have suggested that redox imbalance and oxidative stress are integral parts of ASD pathophysiology (Bjørklund *et al.*, 2020). Early assessment and treatment of antioxidant status may result in a better prognosis as it could decrease the oxidative stress in the brain before it can induce more irreversible brain damage. However, the full comprehension of the role of ROS in ASD is still far to be elucidated. It was noted that oxidative stress can be attributable to the genotoxic effects of ROS, which can causebase modifications and genetic instability. Oxidative stress could induce an alteration in the methylation status of DNA/RNA, mainly by affecting the function and activity of the enzymes responsible for maintaining the epigenetic status, such as methyltransferases, histone methylase, and histone deacetylase. Additionally, it is worth underlining that children with autism have abnormal plasma levels of metabolites in pathways of folate-dependent methionine (transmethylation) and glutathione (transsulfuration) metabolism relative to unaffected age-matched control children (Melnyk *et al.*, 2012). Specifically, cellular methylation capacity expressed as the mean ratio of the methyl donor S-adenosylmethionine (SAM) to the methylation inhibitor, S-adenosylhomocysteine (SAM/SAH ratio), was significantly reduced in many children with autism. A decrease in the SAM/SAH ratio has been associated with the hypomethylation of DNA, RNA, proteins, phospholipids, and neurotransmitters with functional consequences in terms of gene expression, protein expression, membrane phospholipid composition, and dopamine synthesis, respectively. Based on the critical role of the redox and methylation status, it is, therefore, relevant to define biomarkers and show evidence of a functional impact on epigenetic regulation and antioxidant/detoxification capacity in children with autism. The development of tests based on ASD-specific biomarkers would certainly improve the diagnosis of ASD in children. Also, ASD-specific biomarkers may be used to evaluate treatment efficacy as a complement to current behavioral assessment (James *et al.*, 2004; Melnyk *et al.*, 2012). Recent studies show that the assessment of oxidative stress can be based on measurements of antioxidant enzymes and compounds, protein/DNA oxidation, and lipid peroxidation, such as important potential biomarkers of oxidative stress in ASD, e.g., methionine, cysteine, transferrin, 8-hydroxy-2'-deoxyguanosine, ceruloplasmin, 3-chlorotyrosine, 3-nitrotyrosine, F2-isoprostanes, and compounds in the glutathione system (Ranjbar *et al.*, 2014; Kałużna-Czaplińska *et al.*, 2017; Glinton & Elsea, 2019; Osredkar *et al.*, 2019). Measurements of reduced glutathione concentration, glutathione/glutathione disulfide ratio, or homocysteine thiolactone concentration provide information on whether patients are exposed to oxidative stress and whether their cellular antioxidant defense mechanisms work effectively (Kałużna-Czaplińska *et al.*, 2017; Gątarek *et al.*, 2020).

In the previous study (Bobrowska-Korczak *et al.*, 2019), the levels of 6 modified nucleosides/bases such as guanosine, adenosine, guanine, and adenine nucleotides were studied. A group of 22 children with ASD and 20 neurotypical children participated in the study. The results showed significantly lower levels of 7-methylguanosine, 1-methyladenosine, 1-methylguanine, 7-methylguanine, and 3-methyladenine in the urine of children with ASD compared to neurotypical children. These preliminary results show that modified compounds suggest metabolic disorders and nutritional deficiencies in ASD children. The same nucleosides/bases in a larger group of 143 ASD children and 68 matched neurotypical controls were further analyzed to confirm these results. These 7 metabolites were selected as being considered the major physiological catabolites of human purines and pyrimidine metabolism in these subjects (Micheli *et al.*, 2011; Fumagalli *et al.*, 2017). The present study aims: (1) to determine the concentrations of O-methylguanosine, 7-methylguanosine, 1-methyladenosine, 1-methylguanine, 7-methylguanine, 8-hydroxy-2`-deoxyguanosine, and 3-methyladenine in the urine of children and adolescents with ASD in comparison to their neurotypical peers; (2) to check if the results of modified compounds differ depending on the level of autistic-mediated deficit (mild, moderate or severe). Due to the small size of the severe deficit group, these results were not further considered.

MATERIALS AND METHODS

Participants

The study group included 143 children. Sample size calculations, to reach the minimal significant sample size for two independent study groups, with anticipated incidence (endpoint) of 20% of subjects with ASD and impairments in the purine and pyrimidine metabolism, according to Gevi and coworkers (Gevi *et al.,* 2016), with statistic power=80% α=0.05, β=0.2 gave the minimal number of 68 patients, 34 individuals with ASD and 34 neuro-typical controls. In the recruited samples, the subjects' average age was 9.5 years in the range of 2.1-18.1 years. The control group included 68 neurotypical children without any acute or chronic illness, who were, on average, 8.3 years of age in the range of 2.5–20.8 years. Children in the study group were diagnosed with ASD by an expert paediatrician or a neuropsychiatrist in collaboration with a psychologist (Supplementary Material Table S1). The diagnosis was made using a multidisciplinary approach which combined a clinical evaluation with a psychological assessment. Children were grouped according to the criteria detailed and summarized by DSM-5 (American Psychiatric Association, 2013; Kuhn *et al.*, 2018). Additional behavioural ratings were based on a standardized classification of behaviour for children with ASD developed by the local educational authority for providing additional school support (Chawla *et al.*, 2002; Vovk-Ornik, 2015; Osredkar *et al.*, 2019). Ratings were given for two separate dimensions: a) the presence of deficits in social communication and social interaction, and b) the presence of deficits in behavioural flexibility and limited interests and activities. Each child in the ASD group received a rating on each of the two dimensions on a three-point rating scale (1: mild deficit; 2: moderate deficit; 3: severe deficit). Children in the control groups received a rating of 0 for both dimensions. Children with other additional diagnoses were excluded from the study. The children with ASD were not on a gluten-free, casein-free, or sugar-free diet.

The demographic characteristics of participating children and adolescents are given in Table 1. The study

Table 1. Demographic characteristics of autism spectrum disorder (ASD) and neurotypical control group.

	ASD	Control
N	143	68
Male	88%	60%
Median Age (in years)	9.5	8.3
Age range (in years)	2.1–18.1	2.5–20.8
Deficits in social communication		
Mild	47%	/
Moderate	47%	/
Severe	6%	/
Deficits in behavioural flexibility		
Mild	48%	/
Moderate	43%	/
Severe	9%	/

protocol was approved by the National Medical Ethics Committee (0120-201/2016-2 KME 78/03/16).

Analytical Methods

Second-morning urine specimens from all participants were collected. Urine samples were aliquoted immediately after collection and frozen at temperature of –80°C until they were analyzed. Before analysis, urine was thawed and centrifugated.

Modified nucleobases and nucleosides were determined using liquid chromatography coupled to mass spectrometry (LC-MS/MS) on Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, US) coupled to QTRAP 4000 (AB Sciex, Framingham, MA, US). The method was validated and described previously (Raćkowska *et al.*, 2019). Briefly, MRM transitions, for O-methylguanosine, 1-methyladenosine, 7-methylguanosine, 7-methylguanine, 3-methyladenine, 1-methylguanine, and 8-hydroxy-2`-deoxyguanosine tubercidin (internal standard) were (*m/z*): 298->152 (collision energy, CE=17 V), 282->55 (CE=87 V), 298->166 (CE=19 V), 166->79 (CE=43 V), 150->123 (CE=31 V), 166->135 (CE=31 V) and 267->135 (CE=27 V), respectively. The curtain gas, ion source gas 1, ion source gas 2, and collision gas (all high-purity nitrogen) were set at 241 kPa, 207 kPa, 345 kPa, and "high" instrument units, respectively. The ion spray voltage and source temperature were fixed at 5500 V and 600°C, respectively. SeQuant® ZIC®-HILIC column (50 mm×2.1 mm; 5 µm) obtained from Merck (Darmstadt, Germany) was used for chromatography. The gradient of 20 mM ammonium acetate (eluent A) and acetonitrile with 0.2% formic acid (eluent B) presented as (%B) was as follows: 0 min, 95%; 1 min, 95%; 7 min, 50%; and 8 min, 50%. Second-morning urine was collected from all the participants, aliquoted, immediately frozen, and stored at -80°C till the analysis. On the day of the analysis, the sample was allowed to thaw. The centrifuged urine sample (5 min at 1000×*g*, 0.1 mL) was mixed with the tubercidin (0.1 mL, 1 µg/mL) and shaken for 3 min at high speed. Then, 0.6 mL of ice-cold acetonitrile was added, and the samples were vortexed for 3 min and centrifuged (5 min at 10 000×*g*). Five µL of supernatant was injected into LC-MS/MS.

The analysis was performed following the Guideline on bioanalytical method validation of European Medicines Agency guidelines in its work. Thus, together with the study samples we analyzed the blank sample (processed matrix sample without analyte and internal standard), zero sample (processed matrix with internal standard), and quality control samples (at low, medium, and high concentrations) at the level of at least 5% of the number of study samples.

The level of the modified nucleosides/bases in urine was standardized by conversion to the creatinine level.

Quantitative determination of creatinine concentration in urine was performed on a Roche Modular P analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical Analysis

Statistical analysis was performed using Statistica 9.0 software (StatSoft, Poland STATISTICA, version 9.0, Quest Software, Aliso Viejo, CA, USA). The normal distribution of the data was tested using the Kolmogorov-Smirnov test. In the case of normal distribution and homogeneity of variance, a one-way ANOVA was used, while in the case of non-normal distribution, median values were calculated following the Mann-Whitney U non-parametric test. The results were considered statistically significant when $p<0.001$. Spearman's Rank Correlation Coefficient was used to verify if the results correlate to the disorder's severity.

RESULTS

Table 1 presents the demographic characteristics of autism spectrum disorder (ASD) and neurotypical control groups showing that the samples' statistical stratification is homogenously dispersed.

One of the study's major objectives was to examine the levels of O-methylguanosine, 7-methylguanosine, 1-methyladenosine, 1-methylguanine, 7-methylguanine, 8-hydroxy-2'-deoxyguanosine, and 3-methyladenine in the urine of children and adolescents with ASD in comparison to their neurotypical peers. The distribution of urine concentrations of a selected representative group of metabolites, referred to excreted creatinine as ng/mg and µg/mg, was not normal if one considers their own different ability in metabolizing nucleotides (intraindividual variability). Curves have skewness and kurtosis values that obliged researchers to use medians as a main comparative variable. Table 2 shows both mean ± standard deviation (S.D.) and median with CI95 of the indicated metabolites. The major observation is that only 2 of 5 measured guanine/guanosine metabolites were higher: O-methylguanosine and 7-methylguanosine in ASD patients compared with neurotypical controls. Children with ASD have about a 20–30% reduction in metabolite excretion, whereas they exhibit a very high 7-methylguanosine excretion (>65%), a modest higher O-methylguanosine excretion (13%), and a quite unchanged (+2%) level of 8-hydroxy-2'-deoxyguanosine (Table 2).

The values in Table 2 are compared according to their medians, but further comparisons conducted by removing the highest outliers (±S.D.) confirmed the observed differences in excreted metabolites between ASD subjects and neurotypical controls. Due to the fact that the Bonferroni correction was applied in the multiple comparison . To determine if any of the 7 correlations is statistically significant, the *p*-value must be $p\leq0.007$. Therefore, we considered the *p*-value≤0.001 to be statistically significant. Due to the high variability in the levels of the determined compounds at this stage of the study, their usefulness as potential biomarkers in ASD cannot be clearly concluded.

Table 2. Statistical evaluations with corresponding *p*values of ASD and control group.

Name of the compound	ASD	Control			
All results are expressed as on mg creatinine	Mean ± S.D. Median (IC95)	Mean ± S.D. Median (IC95)		Delta %[1]	*p*[2]
O-methylguanosine [ng/mg]	913.98±1528.08 434.11 (661.38–1166.59)	494.36±651.34 384.49 (336.70–652.02)	↑	12.90	0.0745
3-methyladenine [ng/mg]	14.09±30.50 6.33 (8.84–19.34)	14.68±17.10 9.11 (10.47–18.88)	↓	30.52	0.0015
1-methylguanine [ng/mg]	460.04±617.12 299.75 (358.02-562.05)	450.25±300.60 421.71 (377.49–523.01)	↓	28.92	0.0084
1-methyladenosine [µg/mg]	48.87±62.58 30.87 (38.53–59.22)	52.99±25.73 45.33 (46.77–59.22)	↓	31.90	$<1.99\times10^{-5}$
7-methylguanine [ng/mg]	55.23±69.55 35.93 (43.74–66.73)	53.24±24.78 45.59 (47.24–59.24)	↓	21.19	0.0002
7-methylguanosine [µg/mg]	136.67±241.70 51.96 (96.71–176.62)	75.40±104.57 44.30 (48.62–102.18)	↑	17.29	0.1035
8-hydroxy-2′-deoxyguanosine [ng/mg]	14.62±22.68 9.46 (10.57–18.67)	11.97±10.13 9.69 (9.48–14.46)	↓	2.37	0.3243

[1] calculated on medians; [2] *p*-values calculated by a Mann-Whitney U test.

The differences with the *p*-value≤0.001 were considered statistically significant. The Spearman's Rank Correlation Coefficient was used to verify if the results correlate to the age (Supplementary Materials Table S4 at https://ojs.ptbioch.edu.pl/index.php/abp/). Only O-methylguanosine concentrations ($p<0.001$) in the control group were significantly correlated with age. No correlations between the other metabolites and age were observed. Scatterplots illustrating the relationship between the age and the concentration of metabolites (A-G) in the urine of ASD patients (Fig. S4 at https://ojs.ptbioch.edu.pl/index.php/abp/) and controls (Fig. S5 at https://ojs.ptbioch.edu.pl/index.php/abp/) were added to the Supplementary Material (https://ojs.ptbioch.edu.pl/index.php/abp/). In the Supplementary Material, (https://ojs.ptbioch.edu.pl/index.php/abp/) plots were provided to illustrate the relationship between the male-female differences in the ASD and control groups. 7-panel (A-G), 4-group scatter plot (mild, moderate, severe, and control) for each of the 7 urinary purines was presented in Fig. S1 (https://ojs.ptbioch.edu.pl/index.php/abp/). The relationship between the gender and the concentration of metabolites in the urine of ASD patients and the concentration of metabolites in the control group were presented in Figs S2 and S3 (https://ojs.ptbioch.edu.pl/index.php/abp/), and also individual differences in the levels of compounds between the groups were found in Fig. S6 (https://ojs.ptbioch.edu.pl/index.php/abp/).

We encountered some significant differences again when examining the relationship between different levels of severity and excreted metabolites. Table 3 shows the proportion of children by group according to the level of deficit. Groups with mild (N=70) or moderate (N=61) impairment are comparable in terms of the number of individuals classified, while the group with severe disabilities (N=12) is small and thus presents a problem for us in interpreting the results. Due to the small size of the severe deficit group, only the results of the mild and moderate deficit groups were compared. Groups were comparable in age 9.4 (mild) and 9.7 (moderate), as well as the male to female ratio.

Table 3. The proportion of patients with a mild, moderate, and severe level of deficit.

	Mild	Moderate	Severe
N	70 (49.0%)	61 (42.6%)	12 (8.4%)
Male %	63 (90%)	54 (88.5%)	9 (75%)
Median age	9.4	9.7	9.9
Age range	4.5–18.1	2.1–17.5	6.1–13.9

The next objective was to verify if the results correlated with the severity of the disorder. The results were checked as to whether metabolites differed depending on the deficit (mild, moderate, or severe). In Table 4, patients were divided into three groups depending on the level of deficit.

This table shows different excreted metabolites related to the level of deficit. As the group with a severe deficit is small, only the results of children from the mild and moderate groups were compared. These data need to be reappraised in the next research study. When comparing median values, taking into account the severity of the disorder (mild and moderate) compared to controls, it can be observed that the values for two compounds (O-methylguanosine and 7-methylguanosine) are similar for both the mild and moderate groups, while they are higher compared to the controls. For all other 5 compounds in the severity groups, the median values are similar but smaller compared to the control group. The Nonparametric Mann-Whitney U test was used to compare the concentration of compounds between the groups. The differences with a *p*-value lower than 0.001 were considered significant. Statistically significant differences between the mild and control groups for 1-methyladenosine (30.5 *vs* 43.51 µg/mg, $p=1.83\times10^{-5}$) and 7-methylguanine (38.41 *vs* 45.11 µg/mg, $p=0.0009$) were observed. For all other determined compounds, no statistically significant difference was found, with a p-value greater than 0.001. In the moderate group, a statistically significant difference in the case of 7-methylguanine (34.09 *vs* 45.11 µg/mg, $p=0.0007$) was found. It was not statistically significantly different between the mild and moderate groups with a *p*-value greater than 0.001, while statistically significant differences between the ASD and control groups for 1-methyladenosine ($p=1.99\times10^{-5}$) and 7-methylguanine ($p=0.0002$) were observed. All results are expressed on creatinine to eliminate the impact of fluid intake.

Table 4. The statistical distribution of excreted metabolites and severity level of deficit.

	ASD						Control
Compound	Mild	*p*	Moderate	*p*	**p*	Severe	Median
	Median (range)		Median (range)			Median (range)	(range)
O-methylguanosine [ng/mg]	444.51 (44.51–12586.37)	0.0605	432.62 (80.82–3450.42)	0.2376	0.5213	447.59 (14.26–5164.59)	369.90 (125.24–5336.67)
3-methyladenine [ng/mg]	6.66 (0.60–288.69)	0.0362	6.21 (0.39–75.42)	0.0015	0.3167	4.86 (0.38–44.94)	9.00 (1.76–90.40)
1-methylguanine [ng/mg]	304.04 (26.32–5830.77)	0.0148	272.64 (41.84–1588.26)	0.0317	0.8881	313.32 (14.18–1330.31)	417.39 (54.63–1501.42)
1-methyladenosine [µg/mg]	30.50 (2.07–454.16)	1.83×10^{-5}	31.82 (4.97–203.21)	0.0011	0.8917	42.12 (1.34–150.07)	43.51 (12.13–155.20)
7-methylguanine [µg/mg]	38.41 (3.84–568.44)	0.0009	34.09 (8.22–261.10)	0.0007	0.4287	44.30 (1.72–153.09)	45.11 (13.34–140.60)
7-methylguanosine [ng/mg]	59.67 (4.20–1798.71)	0.0454	44.36 (4.22–838.80)	0.5341	0.1593	92.95 (5.34–307.56)	40.43 (3.66–1798.71)
8-hydroxy-2′-deoxyguanosine [ng/mg]	9.96 (0.85–68.23)	0.5086	8.16 (0.89–55.49)	0.1058	0.8662	15.29 (4.58–43.10)	9.56 (0.85–168.23)

**p* Mann-Whitney U test *p*-value for the comparison of Mild and Moderate groups.

Finally, the Spearman's Rank Correlation Coefficient was used to verify if the results correlate to the severity of the disorder. The *p*-value≤0.001 was considered to be statistically significant. No correlations between the severity of the disorder and the levels of metabolite concentrations were observed (Supplementary Materials Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). Nonparametric correlation analysis showed a strong and moderately positive correlation between the concentrations of metabolites determined in ASD urine (Supplementary Materials Table S3 at https://ojs.ptbioch.edu.pl/index.php/abp/).

To accurately demonstrate the biological variation among children with ASD, appropriate graphs are included in the supplementary material (Fig. S1–S6 at https://ojs.ptbioch.edu.pl/index.php/abp/).

Receiver operating characteristic (ROC) curves were used to characterize the diagnostic accuracy and evaluate the predictive accuracy. To obtain a final diagnostic score, ROC curves were generated using the MetaboAnalyst 5.0 (http://www.metaboanalyst.ca). ROC curve showed diagnostic values for 7 urinary purine results classified as mild, moderate, or severe severity of the disorder in ASD compared to controls. The area under the curve (AUC) was used to measure the overall degree of identification power. By combining the all metabolites, the AUC of the ROC curve reached 0.856 (95% CI 0.776 to 0.937) for mild severity of the disorder, 0.838 (95% CI 0.744 to 0.927) for moderate severity of the disorder, and 0.669 (95% CI 0.391 to 0.885) for severe severity of the disorder. The results of the AUC analysis stratified by severity of the disorder were shown in Fig. S7 at https://ojs.ptbioch.edu.pl/index.php/abp/.

DISCUSSION

The significant difference in excreted purines (adenine and guanine) with respect to control neurotypical subjects is a clear marker of impaired ASD metabolism (Gevi *et al.*, 2016). The hypothesis based on folate deficiency in individuals with ASD suggests a possible explanation for our results. Folate depletion may reduce adenosine triphosphate (ATP) and then in S-adenosylmethionine (SAM) in neurons during a rapid cellular division. If a methylation defect arises, this might be a potential, etiopathogenetic, causative factor of ASD due to the consequent depletion in purine and, therefore, in SAM biosynthesis (Geryk *et al.*, 2020). Whereas this could explain the reduction in adenine/adenosine metabolites in ASD patients, the increase in guanine/guanosine metabolites can be explained due to the increase in the ATP/GTP exchange to support the energetic demand, following ATP-SAM impairment.

In the current literature, elevated levels of modified nucleosides/bases are usually associated with ASD. The lack of conclusive evidence that ASD is only a genetic disorder suggests that epigenetic factors may impact ASD's susceptibility. Moreover, there is now compelling evidence that gene by environment interactions are important in the etiology of ASD (Gui *et al.*, 2020; López-Tobón *et al.*, 2020). However, there is a lack of knowledge of how environmental factors interact with genetic susceptibilities to confer individual risk for ASD. In normal and pathogenic brain development, a critical gene expression regulatory mechanism is DNA/RNA methylation. The pathogenesis of ASD leads to miscoded epigenetic mechanisms, cellular processes and functions, and altered expression of genes (Melnyk *et al.*, 2012; Keil & Lein, 2016).

The results showed that in contrast to other urinary metabolites, lower concentrations of 3-methyladenine, 1-methylguanine, 1-methyladenosine, and 7-methylguanine in the urine of ASD were observed, confirming an abnormal purine metabolism in ASD children. Additionally, the concentration of the marker of oxidative stress, 8-hydroxy-'2'-deoxyguanosine, was also lower in ASD than in controls, although not at such an exceptional level as other markers. A more complete picture can be observed when children with ASD are rated into three groups: mild, moderate, and severe. A statistically

significant difference between mild and control groups for 1-methyladenosine (p=1.83×10−5) and 7-methylguanine (p=0.0009) was observed. A difference between mild and control groups in the level of 8-hydroxy-'2'-deoxyguanosine (p=0.5086) was observed, but it was not statistically significant again. In the moderate group, statistical differences in the case of only 7-methylguanine (p=0.0007) were found. Furthermore, other metabolites are not statistically different between the mild and moderate groups. However, the lack of statistical significance might result from the high variability in the metabolite's data distribution.

Moreover, the findings indicate that these compounds could play a potential role in the pathophysiology of ASD. Nutrient deficiencies may lead to hypomethylation, where the nutrients are the source of methyl donors. Significantly lower levels of urinary 7-methylguanosine, 1-methyladenosine, 1-methylguanine, 7-methylguanine, and 3-methyladenine were observed in the research on ASD children conducted by Bobrowska-Korczak and coworkers (Bobrowska-Korczak *et al.*, 2019). Alteration of urinary metabolites is related to a purine metabolism disorder in ASD children (Bobrowska-Korczak *et al.*, 2019). Nucleosides and deoxynucleosides are endogenous metabolites excreted from RNA turnover and DNA degradation, respectively (Patejko *et al.*, 2018). Because the concentration of RNA nucleosides exceeds DNA nucleosides in the cell by about 10 to 1, most of the measured abnormalities are reported as coming from modified mRNA, rRNA, and tRNAs. 7-methylguanosine comes from mRNA. This suggests that capped mRNAs turn over more in ASD patients than in controls. 1-methyladenosine is most commonly found in tRNA and it also is an important regulator of mRNA efficiency. Studies have shown that the up-regulated genes modified by N-6-methyladenine are mainly related to neuron differentiation, neurogenesis, and cell proliferation (Zhou *et al.*, 2020). N-6-methyladenine is the most abundant methylation modification of mRNA in eukaryotic cells, which affects every process of the RNA life cycle. Alteration in the purine metabolism was also observed in the research on ASD children conducted by Bitar and coworkers (Bitar *et al.*, 2018). They found that 5-aminoimidazole-4-carboxamide, an intermediate metabolite in purine synthesis and guanine, was altered in ASD children's urine. Accumulation of aminoimidazole carboxamide and succinyladenosine in body fluids is caused by adenylosuccinase deficiency, an inborn error of purine metabolism. This is manifested by epilepsy, developmental delay, and ASD. The deficiency may cause neurological problems like developmental delay and intellectual disability in purine nucleoside phosphorylase, which is responsible for the metabolism of purine such as guanine (Markert, 1991; Zecavati & Spence, 2009; Bitar *et al.*, 2018). Other researchers have also studied epigenetic changes in ASD (Goldani *et al.*, 2014; Tang *et al.*, 2017).

Several purine metabolism disorders are linked to ASD or behavioural features (Balasubramaniam *et al.*, 2014). Adenylosuccinate lyase (ADSL) deficiency is also an alteration of purine (adenine) metabolism and is caused by mutations in the ADSL gene. The symptoms of this deficiency are various and include delayed development of mental and movement abilities, problems with communication and social interaction, psychomotor retardation, hypotonia, and seizures. However, it is still unclear whether this alteration of pathological mechanisms results directly from the deficiency of intermediates' purines or toxicity (Spiegel *et al.*, 2006; Micheli *et al.*, 2011).

Adenosine deaminase (ADA) is one of the essential enzymes in purine catabolism, which catalyzes adenosine deamination into inosine, thus having an important role in immunological responses. Examples of ADA neurological deficiency, include hearing loss, seizures, autism-like behavior, learning disability, and attention deficit (Bottini *et al.*, 2001; Kelley & Andersson, 2014). Phosphoribosyl-pyrophosphate synthetase (PRPS) superactivity is a disorder with overproduction and accumulation of uric acid (2,6,8-trioxypurine) in blood and urine. The major manifestations of PRPS are hyperuricemia with gout, sensorineural hearing loss, mental retardation, and hypotonia. Hypoxanthine-guanine phosphoribosyl transferase (HPRT) deficiency is related to recycling the purine bases hypoxanthine and guanine to nucleotides. HPRT deficiency occurs as a full spectrum of residual enzyme activity, from mild to severe. Three major clinical features are connected with HPRT: hyperuricemia, neurologic manifestations, and behavioural disturbance (Kelley & Andersson, 2014). Nucleotidase-associated pervasive developmental disorder (NAPDD) is another kind of disorder in purine metabolism and is related to a ten-fold increase in purine and pyrimidine '5'nucleotidase activity (Page *et al.*, 1997). There are numerous symptoms associated with this disorder. Among them are language delay, behavioural disorders, hyperactivity, attention deficit, aggressiveness, social maladjustment, epilepsy, coordination impairment, ataxic gait, and dexterity problems (Micheli *et al.*, 2011). Some of these symptoms are characteristic of ASD (Abraham *et al.*, 2019).

The research on epigenetic mechanisms connected with RNA/DNA molecules is still under-investigated. At present, there is no clear answer to whether methyl marks of RNA/DNA play any role in ASD (Abraham *et al.*, 2019).

CONCLUSIONS

The results reported in the present study show that modified metabolites can play a potential role in the pathophysiology of ASD and that each compound shows a unique pattern that depends on the degree of deficit. These results showed that in contrast to other urinary metabolites, only 2 of 5 measured guanine/guanosine metabolites were higher: O-methylguanosine and 7-methylguanosine in ASD patients compared to neurotypical controls. Moreover, lower concentrations of 3-methyladenine, 1-methylguanine, 1-methyladenosine, and 7-methylguanine in the urine of ASD children were observed, indicating abnormal purine metabolism in ASD children.

Additionally, the concentration of the oxidative stress marker, 8-hydroxy-'2'-deoxyguanosine, was also lower in ASD compared to controls. A different picture is seen when we rate patients into mild and moderate deficits. A statistically significant difference between mild and control groups for 1-methyladenosine (p=1.83×10^{-5}) and 7-methylguanine (p=0.0009) was observed. The levels of 3-methyladenine, 1-methylguanine, 1-methyladenosine, and 7-methylguanine in the mild and moderate groups were lower than in the control group. These findings must be further confirmed in future studies.

Declarations

Conflicts of Interest. The authors declare no conflict of interest.

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