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-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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INTRODUCTION

Autism spectrum disorder (ASD) is a complex, pervasive developmental disorder with a neurological-epipathogenetic 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; Kalužna-Czaplińska et al., 2014; Żurawcz & Kałużna-Czaplińska, 2015; Kalužna-Czaplińska et al., 2018; Adams et al., 2019; Glin ton & Elsca, 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 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 pre-
disposed individuals (Lambeth, 2007; Melnyk et al., 2012; 
Ranjbar et al., 2014; Kaluzna-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 as 
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 irrevers-
able 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 cause base 
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) me-
tabolism relative to unaffected age-matched control chil-
dren (Melnyk et al., 2012). Specifically, cellular methyl-
ation capacity expressed as the mean ratio of the methyl 
donor S-adenosylmethionine (SAM) to the methylation 
hypotheses of DNA or RNA, proteins, phospholipids, and 
neurotransmitters with functional consequences in terms of gene expression, protein ex-
pression, membrane phospholipid composition, and 
dopamine synthesis, respectively. Based on the critical 
role of the redox and methylation status, it is, therefore, 
relevanta to define biomarkers and show evidence of a 
functional impact on epigenetic regulation and antioxi-
dant/detoxification capacity in children with autism. The 
development of tests based on ASD-specific biomarkers 
would certainly improve the diagnosis of ASD in chil-
dren. 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 oxida-
tive stress can be based on measurements of antioxidant 
enzymes and compounds, protein/DNA oxidation, and 
lipid peroxidation, such as important potential biomark-
ers of oxidative stress in ASD, e.g., methionine, cysteine, 
transferin, 8-hydroxy-2′-deoxyguanosine, ceruloplasmin, 
3-chlorotyrosine, 3-nitrotyrosine, F2-isoprostanes, and 
compounds in the glutathione system (Ranjbar et al., 
2014; Kaluzna-Czaplińska et al., 2017; Glinton & Elsea, 
2019; Osredkar et al., 2019). Measurements of reduced 
glutathione concentration, glutathione/glutathione di-
sulfide ratio, or homocysteine thiolactone concentration 
provide information on whether patients are exposed to 
odioxidative stress and whether their cellular antioxidant de-
fense mechanisms work effectively (Kaluzna-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 re-
results showed significantly lower levels of 7-methylgua-
nosine, 1-methyladenosine, 1-methylguanine, 7-methyl-
guanine, and 3-methyladenine in the urine of children 
with ASD compared to neurotypical children. These 
preliminary results show that modified compounds sug-
gest 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-methylguanamine, 1-methyladenosine, 1-methylguanine, 
7-methylguanaine, 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 de-
pending 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 inci-
dence (endpoint) of 20% of subjects with ASD and im-
pairments in the purine and pyrimidine metabolism, ac-
cording 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 
neurotypical controls. In the recruited samples, the sub-
jects’ average age was 9.5 years in the range of 2.1-18.1 
years. The control group included 68 neurotypical chil-
dren 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 col-
laboration with a psychologist (Supplementary Material 
Table S1). The diagnosis was made using a multidisci-
pinary 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 interac-
tion, and b) the presence of deficits in behavioural flex-
ibility and limited interests and activities. Each child in 
the ASD group received a rating on each of the two di-
mensions 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 chil-
dren and adolescents are given in Table 1. The study
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 \( \leq 0.007 \). 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 homogeneously dispersed.

One of the study’s major objectives was to examine the levels of O-methylguanosine, 7-methylguanosine, 7-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 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.
The differences with the $p$-value $\leq 0.001$ 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 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.

The next objective was to verify if the results correlate 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.

This table shows different excreted metabolites related to the level of deficit. 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 ($34.09\text{ vs }45.11\text{ µg/mg}, p=0.0007$) and 7-methylguanine ($38.41\text{ vs }45.11\text{ µg/mg}, p=0.0009$) 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 ($34.09\text{ vs }45.11\text{ µg/mg}, p=0.0007$) 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 2. Statistical evaluations with corresponding p-values of ASD and control group.

<table>
<thead>
<tr>
<th>Name of the compound</th>
<th>ASD</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.D.</td>
<td>Median (IC95)</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>O-methylguanosine</td>
<td>913.98±1528.08</td>
<td>434.11 (661.38–1166.59)</td>
</tr>
<tr>
<td>3-methyladenine</td>
<td>14.09±30.50</td>
<td>6.33 (8.84–19.34)</td>
</tr>
<tr>
<td>1-methylguanine</td>
<td>460.04±617.12</td>
<td>299.75 (358.02–562.05)</td>
</tr>
<tr>
<td>1-methyladenosine</td>
<td>48.87±62.58</td>
<td>30.87 (38.33–59.22)</td>
</tr>
<tr>
<td>7-methylguanine</td>
<td>55.23±69.55</td>
<td>35.93 (43.74–66.73)</td>
</tr>
<tr>
<td>7-methylguanosine</td>
<td>136.67±241.70</td>
<td>51.96 (96.71–176.62)</td>
</tr>
<tr>
<td>8-hydroxy-2'-deoxyguanosine</td>
<td>14.62±22.68</td>
<td>9.46 (10.57–18.67)</td>
</tr>
</tbody>
</table>

1 calculated on medians; 2 p-values calculated by a Mann-Whitney U test.
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, 0.858 (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 adenine 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 misfolded 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

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound</th>
<th>Compound</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Median (range)</td>
<td>Median (range)</td>
</tr>
<tr>
<td>O-methylguanosine</td>
<td>444.51 (44.51–12586.37)</td>
<td>432.62 (80.82–3450.42)</td>
<td>447.59 (14.26–5164.59)</td>
</tr>
<tr>
<td>[ng/mg]</td>
<td>0.0605</td>
<td>0.2376</td>
<td>0.5213</td>
</tr>
<tr>
<td>3-methyladenine</td>
<td>6.66 (0.60–288.69)</td>
<td>6.21 (0.39–75.42)</td>
<td>4.86 (0.38–44.94)</td>
</tr>
<tr>
<td>[ng/mg]</td>
<td>0.0362</td>
<td>0.0015</td>
<td>0.3167</td>
</tr>
<tr>
<td>1-methylguanine</td>
<td>304.04 (26.32–5830.77)</td>
<td>304.04 (26.32–5830.77)</td>
<td>304.04 (26.32–5830.77)</td>
</tr>
<tr>
<td>[ng/mg]</td>
<td>0.0148</td>
<td>0.0317</td>
<td>0.0881</td>
</tr>
<tr>
<td>1-methyladenosine</td>
<td>30.50 1.83×10−5</td>
<td>31.82 1.83×10−5</td>
<td>42.12 1.83×10−5</td>
</tr>
<tr>
<td>[µg/mg]</td>
<td>0.0011</td>
<td>0.0917</td>
<td>43.51</td>
</tr>
<tr>
<td>7-methylguanine</td>
<td>34.09 (3.84–568.44)</td>
<td>34.09 (3.84–568.44)</td>
<td>44.30 (1.72–153.09)</td>
</tr>
<tr>
<td>[µg/mg]</td>
<td>0.0007</td>
<td>0.0427</td>
<td>44.30 (1.72–153.09)</td>
</tr>
<tr>
<td>7-methylguanine</td>
<td>4.20–1798.71 0.0454</td>
<td>4.22–838.80 0.05341</td>
<td>5.34–307.56 3.66–1798.71</td>
</tr>
<tr>
<td>[ng/mg]</td>
<td>44.36 44.36</td>
<td>46.64 44.36</td>
<td>40.43 44.36</td>
</tr>
<tr>
<td>8-hydroxy-2’-deoxyguanosine</td>
<td>9.96 (0.85–68.23) 0.5086</td>
<td>8.16 (0.85–68.23) 0.5086</td>
<td>15.29 (4.58–43.10) (0.85–68.23)</td>
</tr>
<tr>
<td>[ng/mg]</td>
<td>0.0105</td>
<td>0.0958</td>
<td>9.56</td>
</tr>
</tbody>
</table>

*p Mann-Whitney U test p-value for the comparison of Mild and Moderate groups.
significant difference between mild and control groups for 1-methyladenosine \((p=1.83\times10^{-5})\) 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 methylated 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-aminooimidazole-4-carboxamidase, 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 adenosylsuccinate 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). Adenosylsuccinate 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 \((2,6,8\text{-trioxypurine})\) 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 \(5’\text{nucleotide activity}\) (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 \((p=1.83\times10^{-5})\) 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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