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A targeted metabolomic study investigating alterations in urine metabolites of Slovak children with autism spectrum disorders

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Abstract

Autism spectrum disorders (ASD) are neurodevelopmental conditions with increasing prevalence and still not fully explained pathomechanism. Aimed at the clarification of the pathophysiology of this heterogeneous disease and detection of the potential biomarkers, we have focused on the metabolomic analysis of urine samples in patients with ASD and typically developing children.

The targeted metabolomic analysis was carried out using a quantitative LC-MS/MS method performed by combining flow injection mass spectrometry analysis with a LC-MS/MS assay (AbsoluteIDQ p180Kit; Biocrates Life Sciences, Austria).

Our results have shown three altered metabolites in ASD patients ($p < 0.05$). We found significantly higher mean levels of pimelylcarnitine (C7-DC) and glycerophospholipid PCaaC34:1, and significantly lower levels of nonanoylcarnitine (C9) in urine of ASD children compared to healthy controls.

The changes in the acylcarnitine spectrum detected in our study may indicate potential mitochondrial dysfunction and imbalance in fatty acid metabolism, that have previously been reported in ASD. Our results suggest that acylcarnitines could have the potential to become a biomarker for ASD, but their diagnostic sensitivity and disease-specificity need to be investigated in more detail and validated on larger set of patients.

Introduction

During the last decades, the prevalence of autism spectrum disorders (ASD) has significantly increased. ASD refers to a group of neurodevelopmental conditions characterized by a wide range of symptoms, skills, and levels of disability. According to the American Psychiatric Association, ASD is characterized by the core deficits that include persistent impairment in social communication and social interaction and by restrictive and repetitive behavior, activities, and interests (American Psychiatric Association 2013). Symptoms usually appear at the early stage of development and affect the individual's ability to function socially, at school, at work, or in other areas of life. It has been shown that the early diagnosis of ASD and subsequent early intervention are beneficial for children with ASD, and they are more likely to result in a major long-term positive effects on symptoms (Swanson *et al.* 2014, Volkmar 2014, Rotholz *et al.* 2017). Although current treatments vary, most interventions focus on managing behavior and improving social and communication skills to enable optimal social functioning and independence. The diagnosis of ASD is exclusively based on observation using standardized behavioral scales, and on parental interviews. There is no specific biological marker known to confirm the diagnosis of ASD. Moreover, no pharmacological intervention is known to cure this condition. A growing number of research initiatives are focused on identifying potential biomarkers for ASD with the aim of facilitating earlier diagnosis, enhancing diagnostic accuracy, and supporting the development of personalized treatment approaches.

Metabolomics is a technology that allows a simultaneous measurement of hundreds of metabolites in a single biological specimen that opened new insights into the dynamics of metabolism in health and disease. Several sources highlight metabolomics as an "innovative and promising technique" for approaching ASD (Tang *et al.* 2023). It is seen as a way to understand the underlying metabolic dysregulations associated with the disorder. Metabolomics reflects the interplay between an individual's genetics and environment, making it a valuable tool for investigating the complex nature of ASD. The current technologies, such as mass spectroscopy, offer a sensitive tool to investigate human body fluids for metabolite profiles potentially suitable to serve as a biomarker for ASD. Given the complexities of the genetic background of ASD, and gene-environment interactions, metabolomic profiling may provide an alternative path for developing an early diagnosis.

Existing evidence of metabolomic analyses of blood plasma/serum of children with ASD have shown metabolomics patterns compatible with mitochondrial dysfunction, excess gut microbial co-metabolites, or imbalanced metabolic pathways, such as the Krebs cycle (West *et al.* 2014, Wang *et al.* 2016). Many published studies performed on urine samples identified an abnormal composition of urinary solutes in the tryptophan/nicotinic acid metabolic pathway, sulfur and amino acid metabolism, purine and pyrimidine metabolism, gut microbial co-metabolites, as well as metabolic signatures of oxidative stress (Yap *et al.* 2010, Ming *et al.* 2012, Emond *et al.* 2013, Mavel *et al.* 2013, Nadal-Desbarats *et al.* 2014, Noto *et al.* 2014, Dieme *et al.* 2015, Gevi *et al.* 2016, Bitar *et al.* 2018, Liu *et al.* 2019). Urine as a biological material for metabolomic analysis has a great potential. Compared with other body fluids such as blood, where the homeostasis mechanism is active, the urine is a final waste product and reflects well the current condition of the organism (Want *et al.* 2010). Thus it can accommodate subtle and comprehensive changes, especially in the early stages of diseases. Moreover, obtaining urine samples is feasible and noninvasive, and urine samples are straightforward and inexpensive to preserve (Jing & Gao 2018).

In our pilot study, we aimed to shed more light on the pathophysiology of ASD and detection of the potential biomarkers by metabolomic analysis of the urine samples of ASD patients and neurotypical controls free of ASD.

Methods

The study was approved by the Ethics committee of the Comenius University Faculty of Medicine, and the University hospital in Bratislava, Slovakia and it is consistent with the 1964 Helsinki declaration and its later amendments. The informed consent form was signed by both parents (or at least one if both were not available) or caregivers of each child.

The 35 ASD patients (4.8+1.6 years) were recruited and diagnosed using internationally accepted standard diagnostic tools Autism Diagnostic Observational Schedule- Second version (ADOS-2) (Lord *et al.* 2000) and Autism diagnostic interview- revised (ADI-R) (Lord *et al.* 1994) in the Academic Centre for Autism Research at Faculty of Medicine, Comenius University in Bratislava. ADOS-2 consists of 5 diagnostic modules, selected after considering the age of a participant and his/her expressive language quality. All ASD patients

Tab. 1. Basic characteristic of participants of urinary metabolomics

	Number	Sex (Male:Female)	Age (Mean+SD)
ASD patients	35	35:0	4.8±1.6 years
Typically developing children	30	12:18	4.6±1.3 years

were diagnosed in Module 1 which includes individuals older than 30 months, with speech limited to using a few words, without the use of complex phrases or whole sentences.

Exclusion criteria for recruitment were as follows: acute illness, using antibiotics or steroidal and non-steroidal drugs, and presence of a systemic disease or any other psychiatric disorder except ASD. In addition to the ASD patients, 30 age-matched neurotypical controls (4.6+1.3 years) without specific diet were enrolled in (Table 1).

The morning urine samples were collected into sterile containers and stored at -20 °C until further analysis. The endogenous metabolites were analyzed with quantitative LC-MS/MS method performed by combining flow injection mass spectrometry analysis with a LC-MS/MS assay (AbsoluteIDQ p180Kit; Biocrates Life Sciences, Innsbruck, Austria). This validated assay allows for the comprehensive identification and the quantification of a large number of endogenous metabolites, including amino acid (21 amino acids and 21 biogenic amines), glucose (sum of hexoses), markers of fatty acid (40 acylcarnitines) and lipid metabolism (90 glycerophospholipids and 14 sphingolipids). Briefly, urine samples were thawn, centrifuged (5000xg/10min), derivatized by phenylisothiocyanate on solid support for 60 minutes at RT and then extracted by methanol. After the dilution with MPW, samples were transferred to sample vials and analyzed on SCIEX 5500 system (AB Sciex LLC, Marlborough, MA, USA) connected with UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA USA).

The measured metabolites were normalized to the concentration of creatinine in MetIDQ software (Biocrates Life Sciences, Innsbruck, Austria) and subjected to statistical analysis performed in R (RCTEAM 2019) using a bootstrap two-sample t-test of mean difference (10000 bootstrap samples with replacement) (Efron & Tibshirani 1993). Data are presented as means and standard deviations.

Results

Targeted metabolomic analysis of the urine samples from ASD patients and healthy controls detected 83 metabolites as being present in at least half the samples (from all 185 analyzed metabolites) (Supplement 1 at request). Among these, three metabolites showed significant changes ($p < 0.05$) in the ASD patients compared with the neurotypical controls. From 40 analyzed acylcarnitines, the pimelylcarnitine (C7-DC) mean level was significantly higher, and nonanoylcarnitine (C9) mean level was significantly lower in urine of the ASD children compared to the healthy controls (Figure 1A and 1B). From 90 glycerophospholipids, one glycerophospholipid PCaaC34:1 mean levels were found significantly higher in urine from ASD children compared to the healthy controls (Figure 1C).

Discussion

To decipher the complex etiology of ASD, metabolomic studies are proving increasingly valuable for identifying metabolic alterations and potential biomarkers. One key group of molecules under investigation includes acylcarnitines (ACs). While primarily known for their role in mitochondrial fatty acid metabolism, these conjugates of carnitine and fatty acyl groups also have a significant and multifaceted relationship with higher brain functions (Picard & McEwen 2014). Existing literature indicates that changes in urinary ACs have been also observed in individuals diagnosed

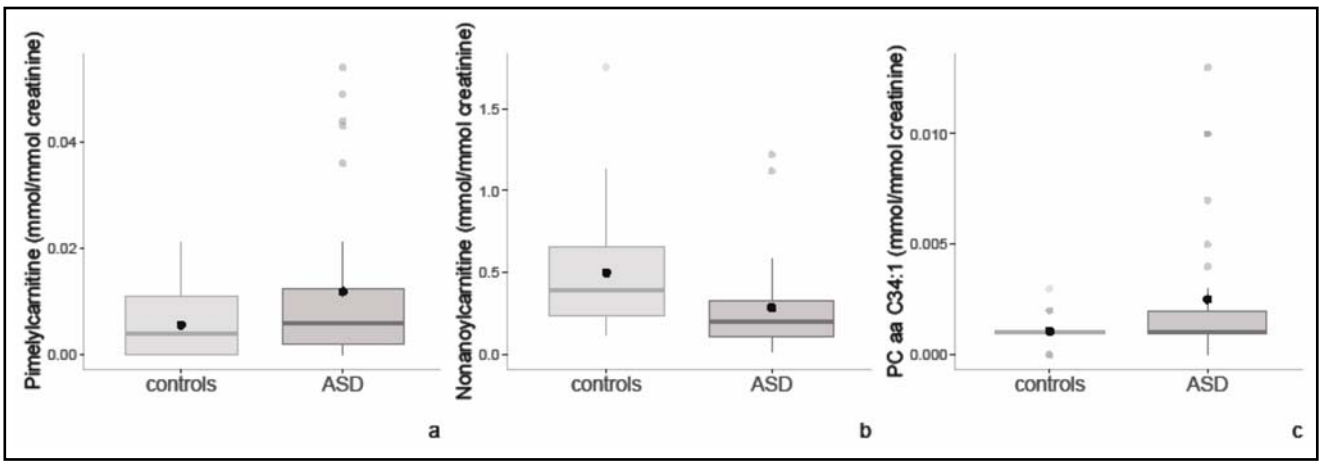

Fig. 1. Urinary levels of Pimelylcarnitine, Nonanoylcarnitine and PC aa C34:1 in ASD patients and age-matched typically developing children.

The endogenous metabolites were analyzed with quantitative LC-MS/MS metabolomics analysis performed by combining direct injection mass spectrometry with a reverse-phase LC-MS/MS. The measured metabolites were normalized to the concentration of creatinine in MetIDQ software and subjected to statistical analysis performed in R using a bootstrap two-sample t-test of mean difference (10000 bootstrap samples with replacement). From 185 analyzed metabolites, three presented metabolites were significantly altered in ASD patients (p-value < 0.05) comparing to the controls. Data are presenting in mmol Metabolite/mmol Creatinine (mean, standard deviation). PC aa C34:1 - glycerophospholipid PCaaC34:1

with autism. The study in Chinese preschool children with ASD has shown significantly lower blood levels of free carnitine, glutaricyl carnitine, octyl carnitine, twenty-four carbonyl carnitine and carnosyl carnitine compared to the typically developing children (Lv *et al.* 2018). Similarly, in the Chinese Han population, L-acylcarnitine and decanoylcarnitine have been differently expressed in the serum of ASD 3-6 years old patients (Wang *et al.* 2016). Significant elevations in short-chain and long-chain, but not medium-chain, ACs were found in the blood of ASD individuals with consistently abnormal acylcarnitine panels (Frye *et al.* 2013b). This pattern of ACs abnormalities was similar to that found in the brain of PPA rodent ASD model (Thomas *et al.* 2010). In spite of the fact that the kit used in our study included 40 ACs ranging from short-chain to long-chain spectrum (from C0 to C18:2), we were not able to detect many of the long-chain ACs in more than 50% of our urine samples (Supplement 1). In our study, differences in two short-chain ACs between the ASD patients and control group were found.

Studies are not uniform regarding differences in individual AC levels between ASD and control samples, which may be caused by differences in protocols, including methods for the analysis of ACs, different statistical methods of data processing, and also by a large variety in genotype, as well as the phenotypic presentation of ASD and therefore differences in behavioral and biological characteristics of the patient groups selected for the study. Differences in detected altered ACs may also depend on the type of biological material used, they also result from the fact that the acylcarnitine profiles in other biological samples, such as the brain or blood are not identically reflected in the urine. This is supported by the study of Fernandez-Ochoa *et al.* who have detected differences between urinary and plasma metabolites, including ACs (Fernandez-Ochoa *et al.* 2019).

In our study, we have found a significant difference between ASD patients and healthy controls in one glycerophospholipid (PCaaC34:1). Glycerophospholipids are a subgroup of phospholipids, the most abundant cell membrane lipids (Osawa *et al.* 2024). Abnormalities in glycerophphospholipid metabolism are implicated in a variety of brain disorders. Alterations in glycerophospholipids and ACs have been also detected and validated in the serum of ASD patients (Wang *et al.* 2016). However, there is insufficient research regarding glycerophospholipids, including PCaaC34:1, in individuals with ASD. Studies examining neuroinflammation, energy and sphingolipid metabolism in the autistic brain suggest that these areas hold promise for ASD biomarker research (Esvap & Ulgen 2023). Nevertheless, further investigations focusing on these metabolites and their connections with a range of behavioral and developmental features associated with ASD need to be performed in well-defined, homogeneous groups of patients with ASD.

The results of Yap *et al.* (Yap *et al.* 2010) indicated significant differences in the urinary pattern of free amino acid, glutamate and taurine. Also, the levels of several amino acids and biogenic amines, such as glycine, serine, threonine, alanine, histidine, glutamyl amino acids, taurine, has been shown significantly altered in ASD children (Ming *et al.* 2012, Mavel *et al.* 2013). Liu *et al.* identified changes in several amino-acids and possible imbalance between excitatory and inhibitory amino acids metabolism in ASD children (Liu *et al.* 2019). In our study, we have analyzed 21 amino acids and 21 biogenic amines included in the kit. Even though we were able to detect 20 amino acids and 17 biogenic amines in more than 50% of analyzed samples, no significant difference between ASD and typically developing children was found.

The important fact is that even though some studies have yielded partially compelling results, as a whole, the findings obtained by metabolomic studies are not consistent and conclusive. There are many factors affecting the metabolites that have been detected as altered in ASD patients. Firstly, the ASD patients are very heterogenous in the degree of autistic psychopathology and behavioral symptoms assessed by standardized protocols, such as the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5, American Psychiatric Association 2013). Therefore, the most promising approach is to cluster ASD patients into homogenous groups according to a measurable marker, which is specific for the individual subgroups and further exploring the metabolome in clinically homogenous groups (Prince *et al.* 2023). Together with the clinical parameters, the sex and the age of ASD patients and the control group play an essential role in metabolomic studies (Gevi *et al.* 2016). These criteria are very important and could be crucial in searching for differences in metabolic patterns of ASD and typically developing children. Next, different methods in metabolites analysis were previously used, including nuclear magnetic resonance spectroscopy (Yap *et al.* 2010, Mavel *et al.* 2013, Nadal-Desbarats *et al.* 2014, Dieme *et al.* 2015) and/or mass spectrometry (Ming *et al.* 2012, Emond *et al.* 2013, Noto *et al.* 2014, Gevi *et al.* 2016). Comparison of the results of different studies opens a statistical challenge given the differences in standardization and statistical analysis between each methodology. In our study, we have analyzed a homogenous set of children diagnosed with ASD in Module 1 (ADOS 2) and age-matched typically developing children by MS. Urinary levels of metabolites were normalized to the concentration of creatinine. In some ASD metabolomic studies, data were normalized by urinary specific gravity or osmolality (Ming *et al.* 2012, Gevi *et al.* 2016), as the authors took into the account a possible reduction of creatinine excretion in ASD patients (Whiteley *et al.* 2006). However, more recent data have shown that there is no statistically significant difference in urinary creati-

nine concentration between the children with ASD and their siblings or unrelated controls (Wang *et al.* 2010). Taking together, metabolomics of urine samples have big potential in identification of new biological markers of ASD. But the standardization of metabolomic analysis and subsequent data processing are very much needed to achieve more conclusive results applicable in ASD diagnosis and treatment.

To conclude, the main components of mitochondrial membranes are glycerophospholipids, and the glycerophospholipid composition is important for function of mitochondria (Dong *et al.* 2023). The membrane functions can be altered as a result of changes in the quality and quantity of phospholipids due to abnormalities of their metabolism (Vanherle *et al.* 2025). Mitochondrial dysfunction may occur as a consequesnce, lead to the occurrence and development of diseases. The main function of L-carnitine is the transport of fatty acids from the cytosol into the mitochondrial matrix, where β-oxidation occurs, and this process leads to esterification of L-carnitine to form acylcarnitines. Thus, the changes in the ACs spectrum detected in our study may indicate potential mitochondrial dysfunction and fatty acid metabolism in ASD. This is in concordance with the evidence that the prevalence of mitochondrial disease and abnormal metabolic markers resulting from impaired mitochondrial function are markedly higher in ASD than in general population (Frye *et al.* 2013a). The possible role of mitochondrial dysfunction in ASD etiology is also supported by a significant correlation between its biochemical markers and the severity of ASD symptoms (Rossignol & Frye 2012). Thus, ACs and glycerophospholipids appear to have the potential to become a biomarker for ASD. However, their diagnostic sensitivity and disease-specificity need to be investigated in more detail, and should be validated on a larger set of patients. Also, the characterization of ACs and glycerophospholipid profiles in different biological materials (brain tissue, blood, and urine) could bring a comprehensive view and help to better understand the role of their different metabolism in ASD etiology. On the other hand, the correlation with the degree of autistic psychopathology and behavioral parameters may be beneficial in terms of phenotypical subgrouping of ASD patients and also of diagnosis of ASD in the future.

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Conflict of Interest

There is no conflict of interest.

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