

Identification of metabolic pathway disturbances using multimodal metabolomics in autistic disorders in a Middle Eastern population

Abstract

We analyzed for the first time the metabolic profile of Lebanese children affected by autistic disorder to compare this profile to other metabolomics studies and to identify the associated metabolic disturbances. Urine samples of 40 patients with Autism spectrum disorder (ASD) and 40 healthy matched controls were analyzed using nuclear magnetic resonance (NMR) and liquid chromatography coupled to high resolution mass spectrometry (LC-MS). Multivariate analysis on analytical data fusion were conducted on the training set of 50 urine samples, and then validated with a test set of 30 samples, the model was also evaluated using a receiver operating characteristic curve. Among the most significant metabolites that contributed to the discrimination between ASD and controls, we confirmed the perturbations of tyrosine, 2-hydroxybutyrate, creatine and glutamate. We found new metabolites such as trigonelline, cysteic acid and guanine. We found metabolic perturbations including amino acids, carbohydrates and oxidative stress pathways which added value for the contribution of known metabolic disturbances in ASD observed in populations of other geographic origins.

Keywords: Autism Spectrum Disorders, targeted metabolomics, fingerprinting, urines, LC-MS, NMR.

Introduction

According to the most recent version of the Diagnostic and Statistical Manual of Mental Disorders DSM-5, autism spectrum disorders (ASD) are a group of neurodevelopmental disorders, characterized by impaired communication and social interaction as well as restricted, repetitive behaviors and activities (1). The prevalence of ASD is estimated to affect about 1 per 160 children, according to the World Health Organization (2) and 1 per 66 children in Lebanon (Chaaya et al., 2016).

To date, the etiology of ASD remains largely unclear despite a huge effort mainly in genetics and neuroimaging. ASD is probably a multifactorial disorder with genetic and environmental factors (3). Several studies have shown metabolic disturbances in individuals with ASD compared to the general population (4,5). Imbalance in the level of certain amino acids in the plasma, platelets, urine or cerebrospinal fluid have been identified in autistic subjects (6). Some of the most common metabolic abnormalities in ASD are those of redox and mitochondrial metabolism (7,8). Furthermore, many neurometabolic disorders such as phenylketonuria, creatine deficiency, altered metabolism of purine, disorders in the metabolism of neurotransmitters and hormones including serotonin, catecholamines, melatonin and GABA (9) have been associated with autism. Metabolomics approach provides metabolic patterns useful to identify biomarkers. There are two approaches in metabolomics: the targeted analysis consisting of the measurement of defined groups of chemically characterized and biochemically annotated metabolites, and the untargeted analysis which is the unbiased comprehensive analysis of all the metabolites in a sample (10). Due to the abundance and the chemical diversity of the metabolites, no single analytical platform can cover the complete range of human metabolome. Analytical platforms frequently used in order to identify and quantify metabolites, are mass spectrometry coupled to separation techniques like gas chromatography (GC-MS) or liquid chromatography (LC-MS) (11) and nuclear magnetic

resonance (NMR). Previous researches have suggested that the study of urine metabolites profiles could be an effective tool for diagnosis and for understanding the physiopathology of autism (12–14). However, most of the studies have focused on Western autistic populations, and as far as we know, none of them have ever taken place on a Lebanese Middle-East population.

The aim of our work is to evaluate the metabolic profile of urine in an ASD Lebanese population by using the complementarity of analytical platforms, NMR 1D (^1H), NMR 2D (^1H - ^{13}C) and LC-MS, in a targeted approach to compare their specific metabolic profiles to previously studied populations, and to identify the metabolic pathways associated with the disorder, for a better understanding of this pathology.

Methods

Sample collection:

Autistic patients were diagnosed and enrolled in the study using Diagnostic and Statistical Manual of Mental Disorders, 4th edition (15) criteria and Childhood Autism Rating Scale (CARS). The average score on the CARS for the patients sample was in favor of moderate autism. The majority of the participants presented with moderate intellectual disability as reported in their records following IQ measures using standardized and validated tools (WISC, WPPSI). Patients were selected through specialized institutions and non-governmental organizations (NGOs) specialized in mental disorders in all the districts of Lebanon. Controls were selected from schools located in all the districts of Lebanon. All families and participants provided informed consent. First morning urine samples were collected from 40 autistic children (30 males and 10 females) and 40 controls (29 males and 11 females) matched for age, gender and geographic location (Beirut, North, South,

Mount Lebanon and Beqaa). All urine samples were centrifuged at 2000g for 5 minutes, the supernatant distributed in volumes of 5 mL and stored at -80°C. An aliquot from each sample was done for creatinine analysis done by the Jaffe method (Olympus AU640, France) to evaluate the dilution effect.

NMR study

Sample preparation

Two mL of urine samples were first lyophilized (FreeZone[®] 4.5 L, Labconco, USA) at -107°C, 0.2 mbar. Then, 300 µL of phosphate buffered deuterium oxide (D₂O) (pH = 7.4 ± 0.5) and 8 µL of an external reference [trimethylsilylpropionic acid (TSP), 0.05 wt % in D₂O] were added. After vortexing and centrifugation for 3 minutes at 1500g, the supernatant was transferred into 3 mm NMR tubes for NMR analysis.

NMR Spectroscopy Analysis

NMR analysis was performed using a Bruker Advance III HD (BrukerSadis, Wissembourg, France) operating at 600 MHz. Spectra were acquired using a “noesypr 1d” pulse program with a relaxation delay of 20s for 1D analysis, and a “Hsqcgpphpr” pulse sequence for 2D (¹H-¹³C) NMR. The region containing the water signal was removed from each spectrum.

Data processing for targeted NMR

¹H-NMR spectra were processed as previously described (16) using TopSpin version 3.2 software (Bruker Daltonik, Karlsruhe, Germany). Spectra were integrated within a range of 0-10 ppm using AMIX software (Analysis of Mixture, version 3.9.14, Bruker, Karlsruhe, Germany), excluding the

water region (4.67-5.00 ppm), with buckets of various width from 0.01 to 0.15 ppm. These buckets (28 buckets) corresponded to single metabolites. The signal intensity in each bucket was normalized to the total sum of peak intensities. Identification of metabolites were achieved using Chenomx software (Chenomx Inc, Edmonton, Canada) and in house database.

For NMR 2D (^1H - ^{13}C), calibration and integration were processed as previously described (16) using MestRenova Software (Mestrelab research, Santiago de Compostela, Spain). The external reference TSP served as reference set at 0 ppm. Each region was integrated manually from several spectra (all cross-peaks presented in 15 controls and in 15 ASD spectra were cumulated). The final 2D matrix contained 542 cross-peaks, then normalized by the total sum of cross-peak intensities and served for statistical analysis. These cross-peaks were then identified using MetaboMiner database (17) and in house database.

LC-HRMS study

Sample preparation

Twenty μL of urine were diluted in water (160 μL) and 20 μL of internal standard (Imipramine) were also added for all urine samples. After vortexing and centrifugation at 10 000 g for 10 min, 150 μL of the supernatant were transferred into a 96-well plate. In order to equilibrate the chromatographic system, twenty quality controls (QCs) [obtained by mixing an equal volume of all urine samples (20 μL)] were injected. The sequence of samples for analysis was randomized, QCs were analyzed every 10 samples and at the end of the run.

LC-HRMS analysis

LC-HRMS analysis was performed as previously described (16), using a UPLC Ultimate 3000 system (Dionex), coupled to a Q-Exactive mass spectrometer operated in positive and negative electrospray ionization (ESI) mode. The system was controlled by X-calibur 2.2 (Thermo Fisher Scientific). UPLC separations were achieved using a Phenomenex Kinetex 1.7 μm XB-C18 (150 mm x 2.10 mm) column. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in methanol. The gradient (16) operated at a flow rate of 0.4 mL/min over a run time of 30 min. During the full-scan acquisition, which ranged from 60 to 900 m/z , the instrument operated at 70 000 resolution ($m/z = 200$).

Data processing for targeted LC-HRMS

A library of standard compounds (Mass Spectroscopy Metabolite Library of standards MS ML[®], IROA technologies) were analyzed with the same gradient of mobile phases and in the same conditions as those used to analyze urine samples. The annotation of selected features were validated using several criteria as previously described (18). Targeted molecules (367 molecules detected in ESI+, 255 in ESI-) were selected and integrated into X-calibur 2.2 (Thermo Fisher Scientific, San Jose, CA). Each peak area was normalized to the total peaks area of each chromatogram (MSTU post acquisition normalization). Coefficients of variation (CV) for QC samples were calculated. Only metabolites with high biological variability were selected. We kept metabolites with CV < 30 % in the QCs and CV > 30 % in the case where the biological variability (CV in samples) exceeded the analytical variability (CV in QCs).

Multivariate analysis

A data fusion model was generated by combining data tables with all features obtained from the four analytical methods (NMR 1D, NMR 2D, LC-MS ESI- and LC-MS ESI+). Data fusion refers to the process of combining data from different matrices for complementary information in order to achieve a global picture of biological samples (19). In this study low-level fusion strategy was used which refers to the concatenation of data matrices (20). Multivariate analyses were performed as previously described (16,21) using SIMCA P+ 13.0 software (Umetrics, Umea, Sweden) which included a principal component analysis (PCA) and an orthogonal partial least squares discriminant analysis (OPLS-DA) using unit variance scaling (UV). The quality of the model was described by the cumulative modeled variation in the X matrix R^2X , the cumulative modeled variation in the Y matrix R^2Y , and the cross validated predictive ability Q^2 values (21,22).

To evaluate the significance of the created model, cross-validation analysis of variance (CV-ANOVA) was applied. The discriminant metabolites named variable importance in projection (VIP) obtained from OPLS-DA, were all considered as responsible for the differences between ASD and control urine samples [from The Human Metabolome Database (HMDB, <http://www.hmdb.ca/>), we only kept in our VIPs metabolites described for their origin as endogenous/microbial]. A Pearson correlation test was performed to eliminate the redundancy of information that correspond to the same metabolites. The redundant metabolite with the lowest p value was kept and the OPLS-DA was rebuilt.

In the first place, we separated our sample based on their age (<8 years and > 9 years). We conducted an OPLS-DA model in order to identify the age-dependent discriminant metabolites. These metabolites were then eliminated for the following analyzes.

Prediction analyses

We randomly split our data into two sets: a training set of 50 samples (25 ASD and 25 matched controls) used to build the OPLS-DA model in order to identify significant features, and a test set of 30 samples (15 ASD and 15 matched controls) used to evaluate the performance of the classification model (see Supporting Information, Table S1) (23). This process, leading to two randomly divided sets, was repeated 10 times, generating new training and test sets each time. From these 10 training sets, we built 10 OPLS-DA models. We created a last OPLS-DA model (model 11) from the common VIP present in at least in 8 of 10 tested models. The performance of this last OPLS-DA model was also evaluated using a ROC curve analysis to evaluate the sensitivity and specificity of the model. Sensitivity is the proportion of “ASD” that were correctly classified as “ASD” and specificity is the proportion of “control” that were correctly classified as “control” (22). The positive predictive value, $PPV = (\text{sensitivity} \times \text{prevalence}) / [\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})]$; and the negative predictive value, $NPV = [\text{specificity} \times (1 - \text{prevalence})] / [(1 - \text{sensitivity}) \times \text{prevalence} + \text{specificity} \times (1 - \text{prevalence})]$. ROC curve was produced using free MetaboAnalyst software (24).

Univariate analysis

Student's *t-test* was performed using MetaboAnalyst (24) for all the features of the total matrix after data fusion in order to select the metabolites which were significant. As we were in a multiple testing case, a non-parametric test was performed with an adjusted *p*-value (set at 0.05) with a False Discovery Rate (FDR) cutoff. The list of all the significant metabolites is given in supplementary material (Supplementary Material, Table S1).

Metabolic pathways

VIP obtained from the final OPLS-DA model and significant metabolites in adjusted t -test (FDR) were introduced in pathways analysis module in MetaboAnalyst based on the latest version of KEGG (Kanehisa et al. 2014), given a metabolic pathway analysis (MetPA). Over Representation Analysis method was a Hypergeometric test. We have focused our interest on pathways showing a p -value <0.05 from the pathway enrichment analysis and deleted those with an impact=0 from the pathway topology analysis.

Cytoscape software (25) was used for visualizing the interactions between the significant metabolic pathways (impact factor > 0 and a p value ≤ 0.05) and the metabolites.

(Insert Fig.1)

Results

Multivariate statistical analysis of the combined $^1\text{H-NMR}$, $^1\text{H-}^{13}\text{C NMR}$, and ESI+/- C18 Column

The general methodology of the study is illustrated in Fig.1. Working with urine requires a normalization treatment to minimize intersample variation due to dilution effect. As previously described (22,26), creatinine normalization did not led to the best multivariate statistical model and/or a better predictive power, so we used a normalization to total peak area (normalization to as constant sum) before statistical analysis. The total number of metabolites from the data fusion was 377 and distributed as shown in the Fig. 2a (NMR1D: 20 metabolites; NMR2D: 60 metabolites; ESI+ C18: 169 metabolites and ESI- C18: 128 metabolites). The complementarity of the chosen analytical platforms is illustrated in Fig.2b.

(Insert Fig.2)

The OPLS-DA scatter plot showed good discrimination between ASD and controls, and the test set $n=30$ (light dots) showed good prediction (only 1 control was misclassified, see Fig. 3a).

The OPLS-DA model gave good statistical parameters: $R^2X=0.348$, $R^2Y=0.897$, $Q^2=0.77$ and $CV-ANOVA=3.7 \times 10^{-12}$. The performance of this model was also evaluated using the ROC curve (27) showing a sensitivity of 80%, specificity of 86%, positive predictive value (PPV) of 8% (taking into account the prevalence of 1/66 in Lebanon) (28), and a negative predictive value (NPV) of 99.7%. Area under the curve (AUC) for the validation set was 0.88 (see Fig. 3b).

(Insert Fig. 3)

The 27 most highly contributing metabolites (VIP), with their fold change, in the discrimination of the 2 populations found in the final OPLS-DA model and the associated metabolic pathways are shown in Table 2.

(Insert Table 2)

To check the relevance of the results, we created from these 27 discriminants metabolites an unsupervised PCA analysis, where we have visualized three different scatter plots in order to confirm that no discrimination was caused by age and gender, compared to patient/control discrimination (Supplementary Material Fig. S1 a-b-c).

To identify the metabolic pathways associated with this autistic population, we created a listing of the significant metabolites coming from univariate analysis ($p<0.05$) and all the VIP from

multivariate analysis. From this list, a pathway analysis (24) was done and Fig. 4 represents the most significant metabolic pathways having a p -value <0.05 and an impact factor >0 .

(Insert Fig. 4)

Discussion

Most of ASD urinary metabolomics studies have been achieved using 1 or 2 analytical platforms (13,35). In the present study, we used a complementary multiplatform analytical approach to improve sensitivity and specificity (16,22) (Fig. 2b, 41% of metabolites coming from NMR and 59% from LC-MS) and to cover the metabolic profile of urine in ASD Lebanese population in order to increase the number of analyzed molecules. Evaluating the performance of the classification model with a test set is important to confirm that the selected features have the robustness to separate the two groups (ASD vs Controls).

We compared our results of potential biomarkers with the findings from other studies of urine metabolic profiles in ASD patients originating from different geographical regions (Table 2). We found perturbations in the urinary metabolites of Lebanese ASD subjects compared to normal children, as previously shown in other studies (14,16,21,22,29–32). However, these studies have shown contradictions. For example, a study conducted on the urine of Italian autistic children (32) has shown perturbations in the metabolism of sugars in ASD subjects, whereas C. Evans *et al.*, reported no significant differences in excretion of sugars between ASD and controls (30). In fact, there are many factors that influence the metabolome and contribute to the variations in human urine metabolic profiles. Some of these factors include diet, different nutritional habits, age, gender, gut microflora and genetics (36). One of the aim of this study is to confirm the previous

metabolomics studies. Some of the biomarkers that we proposed in this study have been already reported in the literature and may represent more robust marker of the disorder (Table 2).

(Fig. 4).

Oxidative stress

Multiple studies have reported evidence of oxidative stress in individuals with ASD (35,38). We found perturbations in metabolites implicated in glutathione metabolism, as well as disturbances in the pathways of cysteine, methionine metabolism and arginine and proline metabolism (3-sulfinoalanine, cysteic acid, creatine, N-acetylalanine) (Fig 4). We also found perturbation of guanidinosuccinate which is produced from oxidation of arginosuccinate by free radicals (39).

Energy metabolism

Creatine deficiency may have a role in the neurobiology of ASD as it plays an essential role in the transfer of energy in the central nervous system. Low levels of creatine have been reported in some regions of the brain of ASD patients (40). Furthermore, deficiency in the levels of acetyl carnitine was found in the Lebanese ASD group compared to controls (See supplementary material, Table S2). Acetyl carnitine is involved in mitochondrial metabolism and plays a role in fatty acid oxidation. Studies have shown that supplementation with L-carnitine improves the behavioral features of ASD (41).

Carbohydrate metabolism

Carbohydrates play an essential role as energy source for cells. Several metabolites implicated in the carbohydrate metabolism appear to be altered in this ASD population. We found perturbations

in the metabolism of propanoate (perturbation in 2-hydroxybutyrate concentration), citrate (perturbation in citrate) and sugars (perturbation in glucose-1-phosphate, fructose, glucuronate and glucosamine).

2-Hydroxybutyrate, an organic acid involved in propanoate metabolism, was found altered in ASD children as previously observed (31). Butyrate is produced by bacteria in the colon and plays a role in the production of energy. Studies have shown that a dietary source of butyrate has a beneficial effects in improving patients with brain disorders (42).

Another metabolite whose level was altered in Lebanese ASD is citrate, already reported in literature (Table 1) (21,22,31,33,34). The primary energy source in the citric acid cycle is acetyl-CoA that derives from the glycolysis of pyruvate *via* pyruvate dehydrogenase. Pyruvate dehydrogenase deficiency is a rare neurometabolic disorder which can lead to neurological problems associated with development of intellectual disability and seizures (43).

Amino acid metabolism

On the other hand, our results showed altered urinary amino acid excretion (Table 1). The concentration of amino acids can be affected by many factors such as age, gender, developmental stage, diet, food habits (44)... However, the cause of fluctuations between studies is still unclear. Our results showed that several amino acids such as serine, glutamate, tyrosine, threonine were altered in Lebanese ASD, as previously described (5). For example, N-acetyl-L-phenylalanine, a metabolite of phenylalanine, and tyrosine, a product of the hydroxylation of phenylalanine, were found to be different in urines of Lebanese ASD. These pathways are greatly impaired in several metabolic diseases. L-glutamate is the major excitatory neurotransmitter in the brain, having functions in learning, memory and synaptic plasticity. Studies have shown an important role of

this amino acid in the pathophysiology of ASD (5,45,46). Levels of glutamate have been described to be higher in the brain and plasma of ASD (5,44). The increase in the excretion of glutamate is not clearly understood but it could be due to drugs, diet, vitamin B6 involvement or impairments in glutamate transporters or receptors (44).

Purine metabolism

Our results also showed an alteration in the purine metabolism. 5-Aminoimidazole-4-carboxamide, an intermediate metabolite in purine synthesis, and guanine were found altered in the urines of ASD. It is noteworthy that adenylosuccinase deficiency is an inborn error of purine metabolism, that results in accumulation of aminoimidazole carboxamide and succinyladenosine in body fluids (47). Symptoms include developmental delay, epilepsy and autism (47).

Deficiency in purine nucleoside phosphorylase (PNP), the enzyme responsible for the metabolism of purine such as guanine, could lead to neurological problems including developmental delay and intellectual disability (48).

In this work, we conducted a metabolomics study of urine on a Middle Eastern ASD population, using a training set, confirmed by a validation test set. Even if this study was realized on a small group (but similar to the literature (44)), we confirmed that several metabolites were modified in the same direction in all studies such as serine, creatine, threonine, hydroxybenzoate, glucuronate, hydroxyproline and guanidinosuccinate. These metabolites should be validated as biomarkers by further studies (developing and validating quantitative assays on other cohorts) to be potentially used in the future for improving the diagnosis of ASD. On the other hand, the other metabolites we found, none yet cited in the literature, could open perspective in identifying specific

pathophysiological pathways. We confirmed in a robust way that several metabolic profiles associated with amino acids, purine metabolism, creatine metabolism, intestinal microbiote, energy metabolism and oxidative stress could be involved in ASD. These findings led us conclude that it might be a common metabolic profile between Lebanese ASD and other studied population despite the differences in the nutritional habits and the environmental factors between countries.

Figure legends

Figure 1: Workflow showing the methodology of discriminant metabolites and metabolic pathways identifications.

Figure 2 a) Venn diagram showing the number of common metabolites between NMR and LC-MS analyses, and the number of specific metabolites for each platform. **b)** Repartition of the 27 discriminants metabolites present in 80% of the 10 permuted OPLS-DA models.

Figure 3 a) Scatter plot of the final OPLS-DA showing the training and the validation set. For the training set: dark blue dots= patients with ASD (n=25) and dark yellow dots= control patients

(n=25). For the validation set: light blue dots= patients ASD (n=15) and light yellow dots= controls patients (n=15). **b)** Validation set ROC curve.

Figure 4: Metabolites interaction and pathway analysis (Impact factor > 0 and $p \leq 0.05$) based on VIP and significant metabolites between autistic and non- autistic Lebanese children. Green color = metabolites with higher level in ASD urines; blue color= metabolites with lower level in ASD urine.

Table 1. Training and test set characteristics (median age and number of males and females)

	Training set		Test set	
	ASD (n=25)	Control (n=25)	ASD (n=15)	Control (n=15)
Age (years)	8.44 [3-14]	8.2 [3-15]	9.13 [4-15]	9.6 [3-15]
no. of males	16	19	12	12
no. of females	9	6	3	3

Table 1. Metabolites of data fusion OPLS-DA with the corresponding metabolic pathway and comparison with published urine metabolomics studies.

Metabolic pathways	Metabolites	Analytical platform	Differentiation for ASD samples	
			Our findings(Fold change)	Published results
Glycine, serine and threonine metabolism	Threonine	NMR2D	↓ (0.6)	↓(29) ; ↓(30).
	Phosphoserine*	ESI- C18	↑ (2.1)	
	Creatine*	NMR2D	↓ (0.7)	↓(21); ↓(14); ↓(22), ↑(13)
	Serine*	NMR2D	↓ (0.8)	
Phenylalanine metabolism	N-Acetylphenylalanine*	ESI+ C18	↓ (0.7)	↓(21) ; ↑(31) ; ↑(32); ↓(30) ↓(33)
	Tyrosine*	NMR1D	↓ (0.9)	
	Hydroxybenzoic acid*	NMR1D	↓ (0.8)	
Glutamate, Arginine and proline metabolism	Glutamic acid*	ESI- C18	↑ (1.3)	↓(21); ↓(14); ↓(22); ↓(30), ↓(13) ↓(21); ↓(14); ↓(22); ↑(13) ↓(30)
	Creatine*	NMR2D	↓ (0.7)	
	Hydroxyproline*	ESI- C18	↓ (0.9)	
Histidine metabolism	Urocanic acid*	ESI- C18	↓ (0.8)	↑(29)
	Glutamic acid*	ESI- C18	↑ (1.3)	↓(21); ↓(14); ↓(22); ↓(30); ↓(13)
Cysteine and methionine metabolism	Phosphoserine*	ESI- C19	↑ (2.1)	↓(29); ↓(31); ↓(30)
	Cysteic acid*	ESI+ C18	↓ (0.2)	
	Serine*	NMR2D	↓ (0.8)	
Propanoate metabolism	2-Hydroxybutyric acid*	NMR2D	↓ (0.6)	↑(31)
Nicotinate and nicotinamide metabolism	Nicotinamide ribotide*	ESI+ C18	↑ (1.8)	↑(31)
	Trigonelline*	NMR1D	↑ (1.4)	
Citrate cycle (TCA cycle)	Citric acid*	NMR1D	↓ (0.7)	↑(21) ;↑(22) ;↑(31); ↑(34) ; ↑(33)
Purine metabolism	Guanine*	ESI+ C18	↓ (0.3)	↑(31)
	5-AminoImidazole-4-carboxamide	ESI- C18	↑ (2.5)	

Vitamin B6 metabolism	Riboflavin	NMR2D	↑ (1.7)	↑(12)
Others	N-Amidino aspartic acid*	ESI- C18	↓ (0.6)	↓(16)
	(guanidinosuccinic acid)	NMR2D	↓ (0.7)	
	Acetylcarnitine	ESI- C18	↓ (0.6)	
	Methyl acetoacetic acid*	ESI- C18	↑ (1.7)	
	Glycerol-3-phosphate*	ESI- C18	↑ (4.9)	
	Cholic acid*			

^a *p*-value* of univariate analysis indicates significant value < 0.05. ^b↑ indicates higher level in ASD

urines, ↓ indicates lower level in ASD urines. Fold change in brackets: [ASD]/ [control]

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