Short Communication

# A metabolome study on 90 autism spectrum disorder patients by mass spectrometry

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**Abstract** Pathogenesis of autism spectrum disorder (ASD) remains unclear and there are still no proper clinical diagnosis biomarkers or effective medical therapy methods for ASD. Previous studies have implicated physiological and metabolic abnormalities in ASD. Here we performed a metabolome analysis of urine and dry blood spot samples from 90 ASD patients and 90 age-matched controls from China using mass spectrometry. To our certain knowledge, this is the first study investigating ASD patients' urinary and blood metabolites at the same time. Abnormalities in urinary and blood amino acids, organic acids and acylcarnitines showed a similar tendency to the reported physiological and metabolic abnormalities in ASD patients. This thesis aimed at detailing the metabolome information of Chinese ASD patients, hoping to help the study of ASD pathology, quantitative diagnosis or therapy methods.

**Key words:** metabolome, autism spectrum disorder, mass spectrometry, urine, dry blood spot

## Introduction

Autism spectrum disorder (ASD) represents a highly heterogeneous collection of neurodevelopmental conditions characterized by social and communication deficits, stereotypic and rigid patterns of behavior, restricted interests, and unusual sensory processing with onset in early childhood[1]. In the US, about 1 in 68 or 1.5% of 8-year-old patients was identified with ASD in 2012, of which boys were 4.5 times more likely to be identified with ASD than girls[2]. There is no official report on the prevalence of ASD in China. A study in 2007 showed that the estimated incidence of 0−4 years old Chinese patients with ASD was 5.49 per 10000[3].

ASD diagnosis by now is relying on impaired social interaction skills combined with restrictive behaviors[1] or ASD diagnostic interview. Many researchers have been focusing on establishing quantitative diagnostic criteria that could contribute to an early and more accurate ASD diagnosis[4]. Physiological and metabolic abnormalities have been proved in ASD, particularly immune dysregulation or inflammation, oxidative stress, mitochondrial dysfunction and environment toxicant exposure[5]. Metabolome changes can be the clue to explain the pathology of ASD; meanwhile, it can point to potential quantitative diagnosis criteria or treatment methods for ASD.

In this study, 90 ASD patients' metabolomes were analyzed compared to 90 healthy children's as controls. Target metabolites including amino acids and organic acids in urine and amino acids and acylcarnitines in blood were detected based on our lab's inborn errors of metabolism screening method using mass spectrometry.

We aimed at investigating the urinary and blood metabolites changing trends in ASD patients to provide more details on metabolome information for clinical diagnosis of ASD patients, hoping to help the study of ASD pathology, quantitative diagnosis or treatment methods.

## Materials and Methods

*Subjects*

90 Chinese ASD patients diagnosed based on ASD diagnostic interview, and 90 age matched Chinese health con-

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**Table 1. Information of ASD patients and controls**

| | ASD | Control |
|---|---|---|
| Number | 90 | 90 |
| Age (year) | 3.8±1.6 | 2.9±1.7 |
| Range of age (year) | 1.5−8 | 0.1−9 |
| Sex (male : female) | 76 : 14 | 65 : 25 |

trols were analyzed as subjects. All the subjects' urine and dry blood spot samples were sent to our lab for inborn errors of metabolism screening. Details on age and gender by groups are provided in Table 1. All experiments were performed in compliance with the relevant laws and institutional guidelines.

*Urine sample preparation and GC/MS analysis*

For the urine samples analysis, 100 $\mu$L of urine was used. Chemicals and the procedure for sample preparation have been described previously[6]. Treated samples were analyzed using gas chromatography coupled to mass spectrometry (GC/MS). GC/MS (JEOL JMS-Q1000GC) was equipped with Ultra Alloy Capillary Column (30 m×0.25 mm×0.25 $\mu$m). Metabolites' peak identification and analyses were performed using our lab's original routine metabolome data analysis software. Urine metabolites were quantified by their ratio to creatinine.

*Dry blood spot sample preparation and LC/MS/MS analysis*

Sample preparation for amino acids and acylcarnitines is summarized as below. Dried blood spot (DBS) was punched into 3 mm diameter and deposited in a microcell plate. The sample was extracted by dispensing 300 $\mu$L of an extraction solution consisting of a mixture of methanol (200 $\mu$L) and an aqueous solution of 3 mmol/L hydratehydrazine (100 $\mu$L). Internal standards (CIL free acid non-derivative NSK-B1 and NSK-A1), stable heavy isotope analogs of several amino acids, free carnitine and acylcarnitines were also present in the extract solution. The sample was shaken for 25 min at room temperature, transferred to a new plate and dried under Nitrogen flow. The sample was then eluted with 260 $\mu$L of acetonitrile/water (70/30; 0.1% formic acid). The extracted sample was injected into the Shimadzu LCMS-8040. Mass spectral data for the amino acids were acquired through a neutral loss scan of 46 Da in positive mode (CE-15 V), and for the acylcarnitines were acquired through a precursor ion scan of 85 *m/z* in positive mode (CE-25 V). Mass spectra for glycine, arginine, citrulline, ornithine and methionine were acquired through multiple-reaction monitoring (MRM) in positive mode.

*Statistical analysis*

Statistical analyses were conducted using Origin version 2016. Means and standard deviations were computed. Test results were showed as mean±SD. All the statistical comparisons were made by means of independent student *t*-Test. The significance threshold was held at a nominal $p<0.05$.

## Results and Discussion

To our certain knowledge, this is the first study investigating ASD patients' urinary and blood metabolites at the same time. 175 kinds of urine metabolites including amino acids, organic acids, carbohydrates and nucleic acids, as well as 51 kinds of blood metabolites including amino acids and acylcarnitines, were analyzed. This time we only focused on urine amino acids and organic acids combining blood amino acids and acylcarnitines. Table 2 shows urinary amino acids and organic acids, and blood amino acids and acylcarnitines comparison data obtained in this study.

Most urinary amino acids and organic acids showed no significant change between ASD patients and controls. Alanine, glycine, valine, leucine, pyroglutamic acid and N-actylaspartate increased in ASD patients while histidine decreased. Lactate, succinate and malate, which are parts of the tricarboxylic acid cycle, showed lower levels in ASD patients than controls. Pyroglutamic acid (PGA) as a glutamate (Glu) analog and a potential precursor and reserve of Glu increased significantly in ASD patients' urine. Homovanillate and vanillylmandelate, which are metabolites of neurotransmitters, decreased in ASD patients. Meanwhile, some other urinary organic acids, which are involved in many other metabolic pathways, also slightly changed between groups. Whether these changes were meaningful for ASD study still needs support from more diagnosed ASD patients' data and research.

Different with urinary components, 19 of 21 blood amino acids were increased in ASD patients. Free carnitine, short chain and medium chain acylcarnitines including saturation and unsaturation ones have significantly changed between ASD patients and control.

This study showed the metabolome analysis of 90 ASD

**Table 2. Urinary amino acids and organic acids, and blood amino acids and acylcarnitines comparison by groups (—, below detectable limit; *, significantly changed component between groups; urine metabolites unit is ratio of metabolite quantitative ion peak area to creatinine quantitative ion *m*/*z* 329 peak area; blood amino acid and acylcarnitine unit is $\mu$M)**

| Component | Mean±SD | | *p* | *t* statistic |
|---|---|---|---|---|
| | ASD | Control | | |
| Urinary amino acids | | | | |
| *Alanine | 0.68±0.65 | 1.09±1.35 | 0.010 | 2.60 |
| *Glycine | 1.54±1.22 | 2.39±3.27 | 0.022 | 2.32 |
| Sarcosine | 0.051±0.058 | 0.045±0.047 | 0.42 | −0.81 |
| *Valine | 0.12±0.12 | 0.17±0.16 | 0.024 | 2.28 |
| *Leucine | 0.057±0.082 | 0.031±0.052 | 0.011 | −2.55 |
| Proline | 0.023±0.020 | 0.12±0.52 | 0.083 | 1.75 |
| Isoleucine | 0.031±0.035 | 0.034±0.048 | 0.59 | 0.54 |
| Serine | 0.64±0.71 | 0.64±0.97 | 0.98 | −0.023 |
| Threonine | 0.12±0.17 | 0.12±0.13 | 0.88 | −0.15 |
| Methionine | 0.0077±0017 | 0.0097±0.031 | 0.60 | 0.53 |
| *Pyroglutamic acid | 1.32±0.97 | 1.94±1.43 | 8.89E-4 | 3.39 |
| Ornithine | — | — | — | — |
| Phenylalanine | 0.070±0.088 | 0.054±0.073 | 0.19 | −1.31 |
| *N-actylaspartate | 0.063±0.054 | 0.11±0.12 | 0.0010 | 3.34 |
| Lysine | 0.0073±0.024 | 0.0057±0.031 | 0.70 | −0.38 |
| Glutamine | 0.0096±0.049 | — | 0.064 | −1.87 |
| Tyrosine | 0.36±0.40 | 0.42±0.36 | 0.31 | 1.01 |
| *Histidine | 0.48±1.47 | 0.14±0.52 | 0.041 | −2.06 |
| N-actyltyrosine | — | — | — | — |
| Cysteine | — | — | — | — |
| Homocystine | — | — | — | — |
| Homoserine | 0.0064±0.047 | 0.0067±0.030 | 0.96 | 0.046 |
| *Phenylalanine/Tyrosine | 0.20±0.12 | 0.16±0.12 | 0.029 | −2.20 |
| *Leucine/Alanine | 0.098±0.13 | 0.052±0.086 | 0.0059 | −2.79 |
| Urinary organic acids | | | | |
| *Lactate | 0.15±0.19 | 0.39±0.70 | 0.0017 | 3.23 |
| Glycolate | 0.072±0.063 | 0.071±0.074 | 0.96 | −0.052 |
| Oxalate | 0.0027±0.0040 | 0.0047±0.010 | 0.079 | 1.77 |
| 3-hydroxypropionate | 0.010±0.015 | 0.016±0.034 | 0.13 | 1.52 |
| Cresol | 0.086±0.16 | 0.10±0.17 | 0.55 | 0.60 |
| *3-hydroxyisobutyrate | 0.028±0.027 | 0.048±0.053 | 0.0017 | 3.20 |
| *2-hydroxyisovalerate | 5.37E-4±0.0017 | 0.0046±0.019 | 0.050 | 1.98 |
| 3-aminoisobutyrate | 1.74±2.95 | 1.61±3.34 | 0.79 | −0.27 |
| 3-hydroxyisovalerate | 0.054±0.093 | 0.097±0.19 | 0.055 | 1.93 |
| *2-methyl-3-hydroxybutyrate | 0.017±0.019 | 0.030±0.042 | 0.0094 | 2.64 |
| 2-ethyl-3-hydroxypropionate | 0.0022±0.0048 | 0.0034±0.0060 | 0.15 | 1.45 |
| Phosphate | 6.00±15.54 | 18.34±73.45 | 0.12 | 1.56 |
| *Succinate | 0.0021±0.006 | 0.0098±0.027 | 0.0093 | 2.65 |
| Methylsuccinate | 0.0020±0.0021 | 0.0030±0.0044 | 0.057 | 1.92 |
| Fumarate | 0.0014±0.011 | 0.0066±0.032 | 0.15 | 1.44 |
| *Glycerate | 0.026±0.028 | 0.040±0.045 | 0.010 | 2.61 |
| *2,3-dihydroxybutyrate | 0.12±0.089 | 0.16±0.13 | 0.022 | 2.31 |
| 2,4-dihydroxybutyrate | 0.089±0.082 | 0.12±0.13 | 0.058 | 1.91 |

**Table 2. Continued**

| | | | | |
|---|---|---|---|---|
| Urinary organic acids | | | | |
| *3,4-dihydroxybutyrate | 0.16±0.14 | 0.24±0.27 | 0.015 | 2.45 |
| Glutarate | 0.0028±0.0061 | 0.0032±0.0046 | 0.62 | 0.50 |
| 3-methylglutarate | — | — | — | — |
| *3-methylglutarconate | 0.0012±0.0020 | 0.0031±0.0044 | 2.63E-4 | 3.75 |
| *Malate | 0.0013±0.0047 | 0.011±0.037 | 0.019 | 2.38 |
| *Adipate | 0.016±0.026 | 0.044±0.084 | 0.0028 | 3.06 |
| 4-hydroxyproline | 7.54E-4±0.0053 | 0.10±0.62 | 0.12 | 1.58 |
| *Erythronate | 1.10±0.82 | 1.66±1.45 | 0.0017 | 3.21 |
| *2-hydroxyglutarate | 0.052±0.046 | 0.09±0.11 | 0.0040 | 2.93 |
| Phenyllactate | — | 0.0011±0.0082 | 0.21 | 1.26 |
| 3-hydroxyphenylacetate | 0.0073±0.013 | 0.0062±0.013 | 0.56 | −0.58 |
| *4-hydroxyphenylacetate | 0.14±0.15 | 0.20±0.22 | 0.038 | 2.09 |
| 2-aminoadipate | 0.017±0.040 | 0.011±0.028 | 0.20 | −1.28 |
| *3-hydroxy-3-methylglutarate | 0.0031±0.0028 | 0.0059±0.0075 | 0.0013 | 3.30 |
| Suberate | 0.0016±0.0031 | 0.0028±0.0057 | 0.082 | 1.75 |
| *Cisaconate | 0.13±0.16 | 0.26±0.32 | 9.93E-4 | 3.37 |
| *Glycerol-3-phosphate | 0.090±0.093 | 0.13±0.12 | 0.0089 | 2.65 |
| *Homovanillate | 0.025±0.017 | 0.048±0.041 | 4.50E-5 | 4.81 |
| *Citrate | 0.036±0.068 | 0.20±0.54 | 0.0044 | 2.92 |
| Methylcitrate | 0.015±0.029 | 0.022±0.025 | 0.081 | 1.75 |
| Hippurate | 4.06±7.42 | 3.19±6.82 | 0.41 | −0.82 |
| *4-hydroxyphenyllactate | 0.014±0.023 | 0.036±0.073 | 0.0074 | 2.73 |
| Vitamin C | 0.44±1.32 | 0.93±2.55 | 1.62 | 0.11 |
| *Vanillylmandelate | 0.15±0.092 | 0.25±0.19 | 5.96E-6 | 4.72 |
| Galactonate | 0.14±0.25 | 0.24±0.42 | 0.066 | 1.86 |
| *Gluconate | 0.17±0.12 | 0.24±0.21 | 0.013 | 2.21 |
| 4-hydroxyphenylpyruvate | 1.04E-4±7.49E-4 | 4.28E-4±0.0023 | 0.20 | 1.28 |
| *3-hydroxysebacate | 0.0011±0.0026 | 0.0060±0.018 | 0.015 | 2.48 |
| 3-hydroxyhippurate | 0.17±0.29 | 0.15±0.35 | 0.69 | −0.40 |
| 4-hydroxyhippurate | 0.41±0.56 | 0.48±0.61 | 0.43 | 0.79 |
| *Stearate | 0.057±0.11 | 0.20±0.41 | 0.0023 | 3.13 |
| 2-ketoglutarate | 2.96E-4±0.0021 | 0.0015±0.0077 | 0.14 | 1.48 |
| Tartarate | 0.0091±0.036 | 0.024±0.11 | 0.20 | 1.29 |
| Pyruvate | 0.16±0.20 | 0.23±0.35 | 0.10 | 1.64 |
| 2-ketoglutaraminate | 0.088±0.11 | 0.072±0.093 | 0.30 | −1.03 |
| Orotate | 0.0080±0.027 | 0.0047±0.012 | 0.29 | −1.07 |
| Urate | 2.01±2.81 | 2.16±3.67 | 0.76 | 0.31 |
| Sebacate | — | — | — | — |
| *Lactate/Pyruvate | 1.47±1.32 | 2.15±2.01 | 0.0087 | 2.66 |
| Blood amino acids | | | | |
| *Alanine | 135.1±42.61 | 95.99±32.03 | 7.38E-11 | −6.97 |
| *Arginine | 11.75±5.44 | 4.28±3.81 | 2.22E-20 | −10.66 |
| *Aspartate | 46.80±15.89 | 36.41±15.66 | 1.72E-5 | −4.42 |
| *Citrulline | 17.38±4.79 | 11.61±3.16 | 3.05E-17 | −9.54 |
| *Cysteine | 0.95±0.21 | 0.77±0.20 | 2.82E-8 | −5.81 |
| *Glutamine | 40.03±6.15 | 27.88±6.85 | 4.03E-26 | −12.52 |
| Glutamate | 65.02±16.23 | 71.71±31.96 | 0.079 | 1.77 |
| *Glycine | 129.86±42.38 | 91.33±24.41 | 7.22E-12 | −7.48 |

**Table 2. Continued**

| | | | | |
|---|---|---|---|---|
| Blood amino acids | | | | |
| Homocysteine | 9.65±4.99 | 8.99±4.08 | 0.33 | −0.98 |
| *Histidine | 53.91±15.83 | 36.34±9.02 | 5.70E-16 | −9.15 |
| *Leucine | 85.96±21.58 | 61.86±17.65 | 5.53E-14 | −8.20 |
| *Methionine | 18.49±4.99 | 14.75±4.44 | 3.36E-7 | −5.30 |
| *Ornithine | 34.34±11.44 | 24.04±6.76 | 1.37E-11 | −7.35 |
| *Phenylalanine | 54.59±12.10 | 40.45±9.34 | 1.95E-15 | −8.77 |
| *4-Piperidinepropionate | 266.0±164.1 | 181.5±96.36 | 4.41E-5 | −4.21 |
| *Proline | 424.7±128.5 | 349.8±147.2 | 3.66E-4 | −3.63 |
| *Serine | 69.37±32.66 | 41.38±12.34 | 8.72E-12 | −7.61 |
| *Tryptophan | 8.74±1.65 | 5.45±1.54 | 6.14E-30 | −13.81 |
| *Tyrosine | 41.02±9.59 | 29.60±9.27 | 7.41E-14 | −8.12 |
| *Threonine | 39.71±12.72 | 27.91±9.66 | 5.85E-11 | −7.01 |
| *Valine | 153.9±35.36 | 107.4±30.80 | 3.10E-17 | −9.40 |
| Phenylalanine/Tyrosine | 1.36±0.30 | 1.44±0.37 | 0.14 | 1.49 |
| Leucine/Alanine | 0.68±0.21 | 0.68±0.21 | 0.84 | 0.21 |
| Blood acylcarnitines | | | | |
| *Free carnitine (C0) | 24.71±6.25 | 19.74±5.64 | 8.33E-8 | −5.59 |
| *Acetyl- (C2) | 19.89±5.26 | 16.76±7.33 | 1.21E-3 | −3.33 |
| *Propionyl- (C3) | 1.80±0.73 | 1.46±0.58 | 8.13E-4 | −3.41 |
| *Isobutyryl-/butyryl- (C4) | 0.16±0.05 | 0.13±0.06 | 2.36E-5 | −4.35 |
| *Isovaleryl-/2-methylbutyryl- (C5) | 0.21±0.10 | 0.12±0.04 | 6.34E-14 | −8.47 |
| *Hexanoyl- (C6) | 0.04±0.02 | 0.03±0.01 | 1.51E-5 | −4.46 |
| *Octanoyl- (C8) | 0.10±0.05 | 0.06±0.03 | 1.46E-9 | −6.41 |
| *Decanoyl- (C10) | 0.23±0.11 | 0.14±0.08 | 2.70E-8 | −5.85 |
| *Dodecanoyl- (C12) | 0.08±0.03 | 0.06±0.03 | 5.86E-3 | −2.79 |
| Tetradecanoyl- (C14) | 0.06±0.03 | 0.06±0.03 | 0.76 | −0.30 |
| Hexadecanoyl- (C16) | 1.11±0.38 | 1.01±0.41 | 0.07 | −1.82 |
| *Octadecanoyl- (C18) | 0.54±0.17 | 0.49±0.15 | 0.05 | −2.01 |
| *Tiglyl- (C5:1) | 0.02±0.01 | 0.01±0.01 | 6.42E-3 | −2.76 |
| *Octenoyl- (C8:1) | 0.17±0.10 | 0.13±0.07 | 3.53E-4 | −3.65 |
| *Decenoyl- (C10:1) | 0.11±0.06 | 0.07±0.04 | 2.39E-7 | −5.39 |
| *Decadienoyl- (C10:2) | 0.04±0.01 | 0.03±0.01 | 0.02 | −2.40 |
| *Dodecenoyl- (C12:1) | 0.04±0.02 | 0.03±0.02 | 9.82E-5 | −3.99 |
| *Tetradecenoyl- (C14:1) | 0.08±0.02 | 0.05±0.02 | 1.04E-14 | −8.45 |
| *Tetradecadienoyl- (C14:2) | 0.02±0.01 | 0.01±0.01 | 2.38E-3 | −3.08 |
| Hexadecenoyl- (C16:1) | 0.10±0.03 | 0.09±0.03 | 0.17 | −1.37 |
| *Octadecenoyl- (C18:1) | 0.40±0.12 | 0.36±0.11 | 0.05 | −1.99 |
| *Octadecadienoyl- (C18:2) | 0.19±0.06 | 0.17±0.06 | 5.36E-3 | −2.82 |
| *Glutaryl- (C5DC) | 0.03±0.01 | 0.03±0.01 | 9.03E-3 | −2.64 |
| 3-hydroxybutyryl- (C4OH) | 0.09±0.04 | 0.09±0.05 | 0.34 | −0.96 |
| *3-hydroxyisovaleryl-/ 2-methyl-3-hydroxybutyryl- (C5OH) | 0.16±0.06 | 0.14±0.05 | 0.05 | −2.00 |
| 3-hydroxytetradecenoyl- (C14OH) | 0.01±0.01 | 0.01±0.01 | 0.10 | −1.67 |
| *3-hydroxyhexadecenoyl- (C16:1OH) | 0.02±0.02 | 0.02±0.01 | 1.10E-4 | −3.99 |
| *3-hydroxyhexadecanoyl- (C16OH) | 0.04±0.05 | 0.01±0.01 | 3.64E-7 | −5.48 |
| 3-hydroxyoctadecenoyl- (C18:1OH) | 0.02±0.01 | 0.01±0.01 | 0.11 | −1.63 |
| *3-hydroxyoctadecanoyl- (C18OH) | 0.01±0.03 | 0.01±0.02 | 0.01 | −2.56 |

patients and 90 age-matched controls from China. Urinary amino acids and organic acids, as well as blood amino acids and acylcarnitines, were compared between these two groups. The abnormal levels of amino acids, organic acids and acylcarnitines in urine and blood of ASD patients showed a similar tendency to reported physiological and metabolic abnormalities in ASD patients. It's worth mentioning that increased urinary PGA in ASD patients is firstly reported this time. There are also issues need further studies like if the increased urinary PGA was meaningful or not in ASD patients and reason for different changing trends of amino acids in urine and blood.

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## Conflict of Interest

The authors declare that they have no competing interests.

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