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Identification of gender-related metabolic disturbances in autism spectrum disorders using urinary metabolomics

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ABSTRACT

Autism spectrum disorders (ASD) are a highly heterogeneous group of neurodevelopmental disorders that are more commonly diagnosed in boys than in girls. The reasons for gender differences in ASD are unknown and no definitive current evidence can explain male predominance. Therefore, in search for laboratory biomarkers responsible for ASD, a comprehensive metabolomics study was performed by metabolic profiling of urine samples in 51 ASD subjects and 51 age- and sex-matched children with typical development. Orthogonal partial least-squares discriminant analysis (OPLS-DA) models with poor quality failed to perform the analysis based on gender in the ASD and control groups. OPLS-DA models based on single-sex samples, especially in female subjects, had better clustering between the ASD and control groups with an increase in the R^2 and Q^2 values compared with those in the whole group. Significantly increased levels of adenine, 2-Methylguanosine, creatinine, and 7alpha-hydroxytestololactone and a decrease in creatine were observed in the female ASD subjects. In particular, 7alpha-hydroxytestololactone, which has a structure similar to that of testolactone, was positively correlated with adenine (Pearson correlation coefficient, $r = 0.738$, $p < 0.01$), creatinine ($r = 0.826$, $p < 0.01$), and 2-Methylguanosine ($r = 0.757$, $p < 0.01$) and negatively correlated with creatine ($r = -0.413$, $p < 0.05$). A receiver operating characteristic curve analysis using the creatinine:creatine ratio yielded an area under the curve of 0.913 (95% CI: 0.806–1). These metabolites may be sex-related or sex-sensitive to an extent and can be valuable for identification of the molecular pathways involved in the gender bias in manifestation of ASD. The creatinine:creatine ratio has a potential to be a good predictor of ASD in the female subjects.

1. Introduction

Autism spectrum disorders (ASD) represent a highly heterogeneous collection of neurodevelopmental conditions characterized by social and communication deficits, stereotypic and rigid patterns of behavior, and restricted interests (American Psychiatric Association, 2013). Recent statistic estimates indicate that the global incidence of ASD has increased to 1 per 100 children (Lai et al., 2014). Despite the lack of good statistical data, it is estimated that there is a large number of autistic children in China because the corresponding special schools and rehabilitation facilities have been significantly expanding during the recent years; the number of new cases will be increasing continuously due to the Two-Child Policy implementation in the near future.

ASD are more commonly diagnosed in boys than in girls; this is a fixed feature of ASD that stays constant despite a significant increase in the global prevalence of ASD in the past decades. The gender ratio of ASD varies depending on the geographical area; the 4:1 gender ratio has been widely cited based on a study that calculated the mean male-to-female ratio in the population prevalence studies of ASD (Fombonne, 2009). A recent systematic review showed that the true male-to-female ratio was closer to 3:1 (Loomes et al., 2017). The reasons for gender differences in ASD are unknown; investigators have attributed the gender bias in manifestation of ASD to the implications of hormones, genetic variations, environmental factors and differential brain growth as well as to the effect of the intra-associated events (Baruah et al., 2018; Kushak and Winter, 2018). However, no definitive evidence currently exists to explain male predominance.

Alterations in glutamate, a primary excitatory neurotransmitter in the brain, have been implicated in ASD (Wickens et al., 2018).

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Bredewold found that male mice had higher levels of glutamate induced by social play compared to that in females, which may be attributed to sex differences in sensitivity to perturbations in the glutamate system (Bredewold et al., 2015). Certain studies hypothesized that the intestinal microbiome may be a factor contributing to the prevalence of ASD in boys based on microbial metabolites and/or epigenetic factors capable of regulating the host gene expression through DNA methylation and/or histone modification (Kushak and Winter, 2018; Stilling et al., 2014). These findings suggested an intriguing hypothesis implying the existence of certain specific metabolites responsible either for a cause or for a consequence of the male predominance.

Metabolomics, a widely utilized bioanalytical methodology in systems biology, employs mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy to define small molecules present in biological samples in response to the genetic, physiological, and environmental changes. Numerous recent studies based on metabolomics have been utilized to search for potential biomarkers of ASD (Bitar et al., 2018; Dieme et al., 2015; Emond et al., 2013; Gevi et al., 2016; Liang et al., 2018; Lussu et al., 2017; Mussap et al., 2016; Nadaldesbarats et al., 2014; Repiska et al., 2017; Wang et al., 2016; Xiong et al., 2016). However, the gender effect has been very rarely investigated independently in these studies, partly due to the small sample size of female individuals, even though metabolic changes have been shown to be associated with gender (Psihogios et al., 2008; Slupsky et al., 2007).

Taking into consideration these issues and aiming to maximize the probability of reliable sex differences in metabolite levels, we focused on autistic and unrelated typically developing children (control) tightly matched in age and gender, Han Chinese, and on a city of origin within the country. A nontargeted metabolomics study used high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS). A mass spectrometer equipped with a dual electrospray ionization (ESI) source uses a technology focused on increasing the sensitivity and is suitable for the detection of metabolites in biological fluids. Application of this experimental approach in combination with multivariate statistical analysis has identified urinary metabolites with the most significant differences in their levels in the ASD subjects versus typically developing children and gender-related metabolic changes in ASD.

2. Materials and methods

2.1. Subjects and specimen collection

This prospective study was approved by the Ethics Committee of Hunan Provincial Maternal and Child Health Care Hospital. Informed consent was obtained from the parents of the participants. ASD subjects were recruited from four special schools and rehabilitation facilities in Changsha, China, by referring to their medical records issued by the local medical facilities based on the DSM-IV or DSM-V criteria; only children with strictly defined autistic disorder were enrolled, whereas children with pervasive developmental disorder-not otherwise specified (PDD-NOS) or Asperger syndrome were excluded from the study. Participants with typical development were recruited from a local kindergarten. All subjects were screened via a questionnaire to assess current and past physical illness. Children with known endocrine, cardiovascular, liver or kidney diseases were excluded from the study. Dietary restriction was not required for participation in the study. Subjects less than 3 years old and more than 7 years old (< 3, > 7) formed a very small fraction of the enrolled ASD individuals; hence, subjects within the age range from 3 to 7 years were selected. Therefore, the final study group included 51 ASD subjects (34 male and 17 female) and 51 age- and sex-matched (34 male and 17 female) children with typical development. The age was not significantly different in the total group (mean age 4.855 ± 1.232 VS 5.016 ± 1.018, $p = 0.072$), male subjects (4.794 ± 1.212 VS 5.017 ± 0.977, $p = 0.102$), and female subjects (4.977 ± 1.299 VS 5.016 ± 1.126, $p = 0.438$) in the ASD and control groups, respectively. There was no significant difference in the age of the male and female participants between the ASD and control groups.

The collection and storage of urine samples in the clinic procedures in laboratory were based on a systemic protocol previously described (Yin et al., 2015). Urine specimens were collected from the ASD and control subjects between 9:00 a.m. and 4:00 p.m. The samples were kept on ice, transported to the laboratory within 1 h, and stored at −80 °C.

2.2. Reagents

HPLC-grade acetonitrile was purchased from Merck KGaA (Germany); formic acid, ammonium acetate, uric acid, 5′-methylthioadenosine, creatinine, creatine, and adenine were obtained from J&K Chemical (Beijing, China). All reference solutions for HPLC-QTOF-MS were obtained from Agilent Technologies (Santa Clara, USA).

2.3. Sample pretreatment

The samples were pretreated as described in our previous work with minor modifications(Xiong et al., 2015). Briefly, urine samples (200 μL) were initially treated with 30.0 μL urease (1.2 U/μL) at 37 °C for 30 min to remove interfering urea. Proteins, including added urease, were precipitated with 600 μL ethanol and removed after 4 min centrifugation (3000 r/min). The supernatant was filtered through a 0.22 μm microporous membrane and 5 μL filtrate was injected into HPLC-QTOF-MS for analysis.

2.4. HPLC–QTOF-MS metabolic profiling analysis

LC-MS was performed using a 1290 Agilent HPLC system coupled to an Agilent quadrupole time of flight (QTOF, 6550) mass spectrometer. The separation of metabolites was performed by an Agilent ZORBAX SB-C18 column (3.0 × 100 mm, 1.8-micron particle size) by maintaining column temperature at 45 °C. The mobile phases were A (water with 5 mM ammonium acetate) and B (acetonitrile) in the negative ESI mode and A (water with 0.1% formic acid) and B (acetonitrile) in the positive ESI mode, respectively. A sample size of 5 μL was injected at a flow rate of 0.4 mL/min. The gradient was started with 5% B and followed the gradient program: 5% B (1 min), 5–60% B (1–3.5 min), 60–95% B (3.5–6 min), and 95% B (6–10 min). A post-run time of 4 min was buffered before injection of a subsequent sample. Mass detector was operated using dual electrospray with reference ions of *m/z*112.9856 and 1033.9885 in the negative ESI mode and *m/z*121.050873 and 922.009798 in the positive ESI mode, respectively, to enable continuous internal calibration during the analysis and to ensure accuracy and reproducibility. The main parameters for MS were as follows: gas temperature 250 °C, drying N_2 gas flow rate 14 L/min, nebulizer pressure 35 psig, fragmentor voltage 380 V, skimmer 65 V, sheath gas temperature 350 °C, sheath gas flow 11 L/min, capillary voltage 3500 V, and nozzle voltage (Expt) 1000 V. Targeted MS/MS mode was used to identify potential biomarkers. The collision energy was applied by a fixed value of 10 and 20. A full range mass scan from 50 to 1200 *m/z*with an extended dynamic range of 2 GHz standardized at 3200 was applied. Centroid mode was used for data collection and storage. MS spectra acquisition was carried out in a mass range from 30 to 1200 *m/z*, whereas MS/MS data were obtained from 30 to 500 *m/z*. A quality control sample (QC) obtained by mixing 20 μL of each urine sample was used to validate the method for monitoring the consistency of the HPLC-QTOF-MS system. The QC sample was analyzed 5 times before the test samples in the negative and positive ESI modes to validate the method. Six single ions with different *m/z*were randomly selected according to their retention time to test the repeatability and stability; the relative standard deviations (RSD) of the retention times

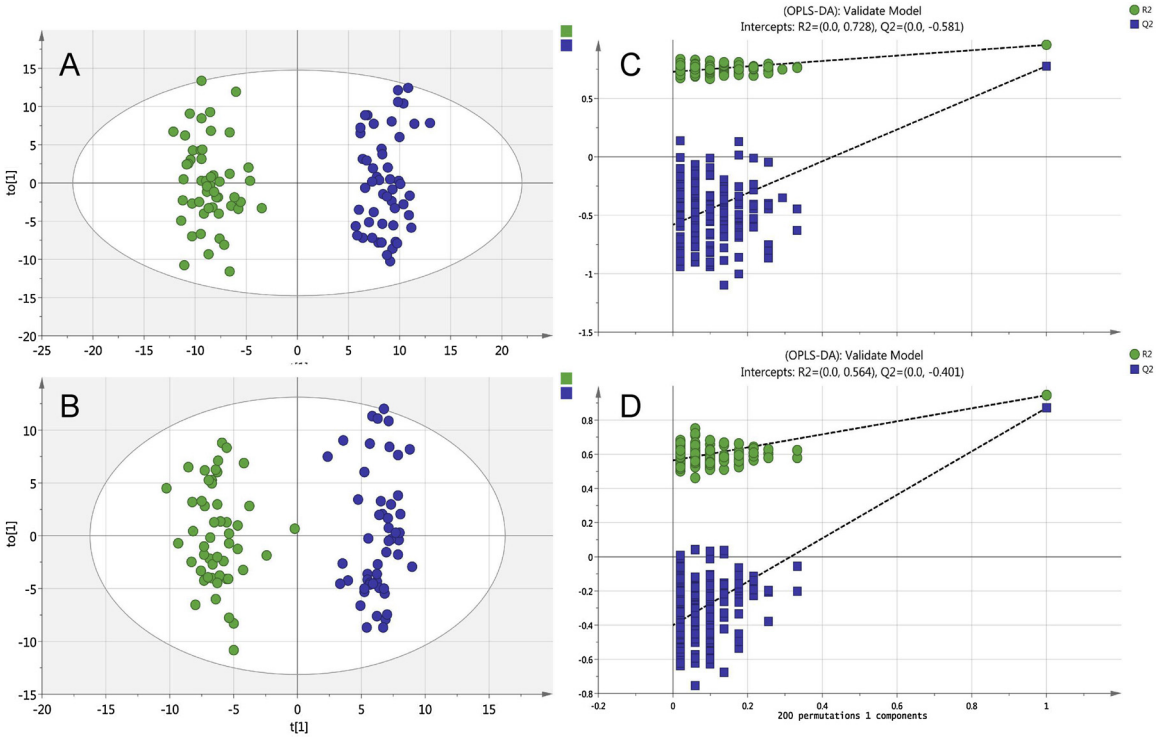

Fig. 1. OPLS-DA models based on overall samples and permutation tests in positive and negative ESI mode. OPLS-DA model based on overall subjects from ASD and controls in positive ESI mode, $R^2 = 0.958$, $Q^2 = 0.777$ (A) and in negative ESI mode, $R^2 = 0.946$, $Q^2 = 0.871$ (B), controls are shown in blue, and ASD are shown in green. Model validation by 200 times permutation test in positive ESI mode (C) and negative ESI mode (D). The 2 points on the right side correspond to R^2 and Q^2 of the observed data set. Other points on the left side correspond to R^2s and Q^2s of permuted data sets. All Q^2 values on the permuted data set to the left are lower than the Q^2 value on the actual data set to the right, indicating that the OPLS-DA modela were valid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and peak areas of these ions were < 12% (data not shown). These results indicate that the present method was suitable for the subsequent analysis of urine samples based on good reliability of the method.

2.5. Multivariate statistical analysis

The data files (".d") obtained from LC-Q-TOF-MS were extracted and aligned using the Agilent Mass Hunter Profinder B08.00 software for a Batch Recursive Feature Extraction (small molecules/peptides) including molecular feature extraction (MFE) and "Find by ion" algorithm. Profinder MFE algorithm extracts the data based on mass spectra and chromatographic characteristics and bins and aligns the features of the compounds; then, these results are used to create a list for a "Find by ion" algorithm. This targeted feature algorithm assists with minimization of the false-positive and negative features detected by the MFE procedure. Files in two types of format, a common format (.csv) and a single data file format (.cef), for each sample were exported. The resulting binning data in the data matrix (.csv), including molecular weight, retention time, *m/z,* and abundance of detected metabolites, were Pareto-scaled (mean centering and scaled to square root of variance) and used in the multivariate statistical analysis by using SIMCA (version 14.1, MKS Umetrics). An orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed to discriminate the metabolic profiles of the ASD and control subjects. The OPLS-DA model was assessed by the intercepts of R^2 and Q^2 in the permutation test to avoid overfitting. The criteria for the model validity included two condition: 1) all Q^2 values of the permuted data set to the left are lower than the Q^2 values of the actual data set to the right and 2) the regression line (line joining the point of observed Q^2 to the centroid of a cluster of permuted Q^2 values) has a negative value of intercept with the Y axis(Mahadevan et al., 2008). Variable importance in the projection (VIP) value of each metabolite was obtained from the OPLS-DA model and the VIP value > 1.0 was retained to select the metabolites that differ between the two groups.

A series of individual ".cef" files were subsequently exported into the Agilent Mass Profiler Professional (MPP, version 13.1.1) software for statistical and differential analysis. The retention time alignment parameter was set to 0.4 min with a mass tolerance of 10 ppm. The data were normalized using a percentile shift algorithm set to 75 and were adjusted to the baseline values of the median of all samples; this procedure can eliminate the bias caused by the weight differences of the initial urine samples(Mayengbam et al., 2016). An unpaired Mann-Whitney *t*-test was employed by the MPP software to evaluate the statistical significance of the metabolites. P-value computation was performed with multiple testing correction by the Benjamini-Hochberg procedure. Differential metabolites that have $p < 0.05$ (corrected) and fold change > 2 in the *t*-test and VIP score > 1 according to OPLS-DA were preliminary selected; only those metabolites that are observed in the male-only or female-only subjects and are involved in the corresponding pathways were finally retained.

2.6. Identification of metabolites

Metabolite identification was based on the results obtained in our laboratory, databases and verification with standards. Potential markers were initially annotated on the basis of their exact mass data (mass error < 5 ppm) and isotopic distributions corresponding to the *m/z*peaks by searching against the Metlin database and Human Metabolome Database (HMDB). Comparison of the structures of the proposed compounds with the fragments obtained in the MS/MS experiments confirmed the identity. Mass spectra, chromatographic retention times and fragmental information of the commercially pure standards were used for the final confirmation.

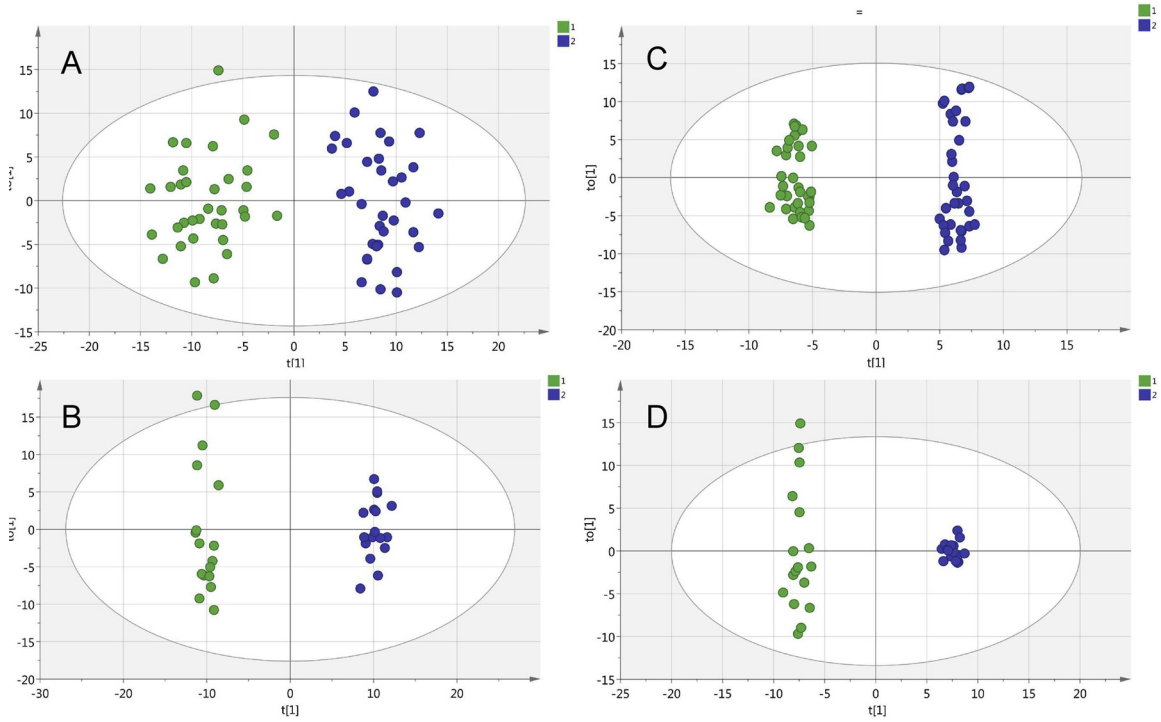

Fig. 2. OPLS-DA models based on male- and female-only subjects in positive and negative ESI mode. In positive ESI mode, OPLS-DA model based on male subjects, $R^2 = 0.903$, $Q^2 = 0.768$ (A) and that based on female subjects, $R^2 = 0.992$, $Q^2 = 0.85$ (B); In negative ESI mode, OPLS-DA model based on male subjects, $R^2 = 0.986$, $Q^2 = 0.921$ (C) and that based on female subjects, $R^2 = 0.993$, $Q^2 = 0.944$ (D). Controls are shown in blue, and ASD are shown in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Multivariate statistical analysis

After extraction by the software and manual verification, a total of 320 and 388 variables were detected across the data set and imported into the multivariate models in the negative and positive ESI modes, respectively. As shown in Fig. 1A–B, the OPLS-DA models with high R^2 (goodness of fit parameter) and Q^2 (goodness of prediction parameter) show distinct separation between the ASD and control groups regardless of gender in the positive and negative ESI modes. The validity of the OPLS-DA models was additionally evaluated as indicated by assessment of the R^2 and Q^2 values that were considerably lower than the original points on the right side (Fig. 1C–D).

The OPLS-DA models based on single-sex studies provide better clustering between the ASD and control groups with increased R^2 and Q^2 values (positive ESI mode, Fig. 2A–B; negative ESI mode, Fig. 2C–D), especially in the female subjects. We hypothesized that intrinsic metabolic variations may exist between the male and female subjects in the ASD group. Nevertheless, OPLS-DA models with poor quality (low R^2 and Q^2) failed to perform based on gender in the ASD and control groups (data not shown). These results suggest that the metabolic changes in the ASD group versus the control group are present and that certain metabolic changes are sex-related or depend on gender to an extent. Thus, to maximize the probability of reliable detection of sex-related differences in the metabolite levels, a list of differential metabolites between the ASD and control groups separated by gender was acquired using the metabolomics investigation of urine samples.

3.2. Identification of biomarkers

Urine marker metabolites were determined by MSMS fragmentation and comparisons with authentic standards if available (see Supplemental materials, Fig. S1). Authentic standards were used to identify uric acid, 5′-Methylthioadenosine, adenine, creatinine, and creatine. The identities of 2-Methylguanosine, N2,N2-Dimethylguanosine, and 7alpha-hydroxytestololactone were tentatively determined through accurate mass-based interpretation of MSMS fragmentation and previous publication on their MSMS spectra (Allen et al., 2015) Finally, 8 most discriminating metabolites (3 in the positive mode and 5 in the negative mode) were identified as summarized in Table 1 with data on exact mass, mass error, corrected p, fold change (FC) and VIP.

3.3. Pathway analysis

The discriminating metabolites were introduced in the pathways analysis module for metabolic pathway analysis (MetPA) in MetaboAnalyst software (version 4.0, http://www.metaboanalyst.ca). Hypergeometric test was selected for over-representation analysis. The MetPA results show that the purine and arginine metabolic pathways are the most perturbed pathways in ASD (Fig. 3).

4. Discussion

Purine metabolites are well represented in the urine of ASD children (Fig. 4). A reduction in uric acid bears an interesting resemblance to that obtained in a patient with ASD features with phosphoribosylpyrophosphate synthetase deficiency(Page, 2000). Urate has antioxidant properties and reduction in urate indicates increased oxidative stress, which has been consistently reported in ASD. As shown in Fig. 4, ASD children preferentially transform adenosine into adenine and 5′-methylthioadenosine, whereas the adenosine-inosine pathway, in which adenosine is converted to inosine by adenosine deaminase (ADA), is less utilized. Interestingly, we found that the adenosine-adenine pathway appears to be more prominent in the female ASD subjects resulting in an increase in adenine in the female subjects. The finding is in line with previous reports that ADA activity is decreased in ASD(Stubbs et al.,

Table 1
Differential metabolites obtained by metabolomics study of urine samples between ASD and controls.

Compound				Male subjects			Female subjects		
Data in positive ESI mode									
Name	Detect mass	Mass error ppm	Regulation	p (Corr)	FC	VIP	p (Corr)	FC	VIP
Creatinine	113.0594	4.4	up				3.17E-06	3.53	1.54
Creatine	131.0701	4.6	down				1.50E-02	−2.82	0.54
7alpha-hydroxy- testololactone	318.1833	0.6	up				6.50E-05	3.11	1.36
Data in negative ESI mode									
Name	Detect mass	Mass error ppm	Regulation	p (Corr)	FC	VIP	p (Corr)	FC	VIP
Adenine	135.0544	−0.7	up				7.47E-10	3.89	1.79
Uric acid	168.0286	1.8	down	1.38E-12	−7.65	1.63	1.81E-09	−11.23	1.66
5'-Methylthioadenosine	297.0889	−2.4	up	1.79E-13	2.57	1.88	1.35E-09	3.56	1.78
2-Methylguanosine	297.1067	−2.0	up				1.48E-09	4.65	1.70
N2,N2-Dimethylguanosine	311.1227	−1.0	up	8.18E-12	2.19	1.80	1.14E-05	2.48	1.63

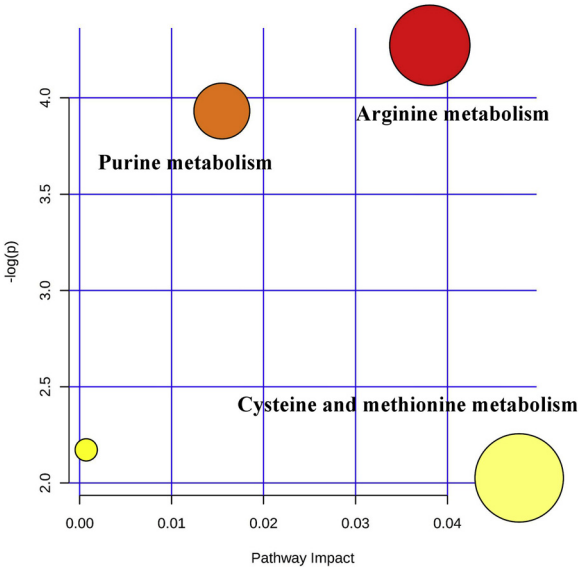

Fig. 3. Metabolic pathway analysis plot. Color intensity (white to red) reflects increasing statistical significance, while circle diameter reflects pathway impact. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

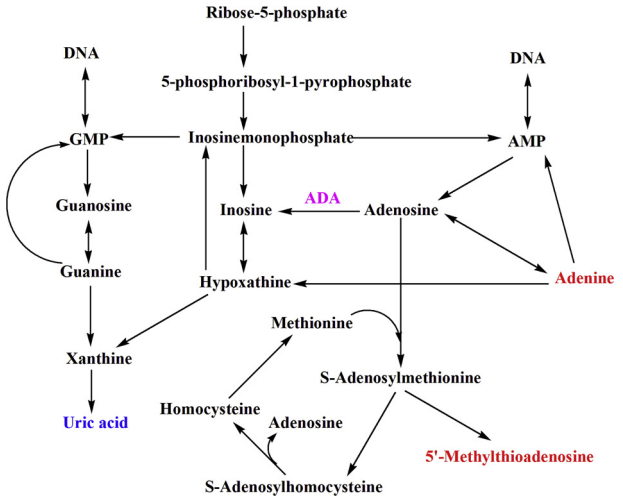

Fig. 4. Metabolic pathway of purine metabolism and methionine cycle. GMP: guanosine monophosphate, AMP: adenosine monophosphate, ADA: adenosine deaminase. Increases of metabolites are shown in red, while decreases of metabolites are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1982), and that the presence of the low-activity ADA allele (ADA2) in autistic cases shows a significantly elevated frequency(Bottini et al., 2001; Persico et al., 2000). Importantly, a disruption of the adenosine-to-inosine pathway in ASD may be linked to adenosine-to-inosine (A to I) RNA editing, a neurodevelopmentally regulated epigenetic modification shown to modulate complex behavior and one of the many mechanisms connecting environmental stimuli and behavioral output (Gatsiou et al., 2018). A to I RNA editing biochemically refers to hydrolytic deamination of adenosine to inosine in double stranded RNA at the presence of ADA acting on RNA (ADAR). Altered inosine metabolism may imply changes in A to I RNA editing. Supporting the hypothesis, a previous study described a high dynamic range of the editing levels of synaptic genes related to ASD. Moreover, a dysfunctional form of the editing enzyme adenosine deaminase acting on RNA B1(pre-mRNA of the glutamate receptor subunit B) was found more commonly in the postmortem cerebella from individuals with ASD compared with that in the case of neurotypical individuals (Eran et al., 2013). This study suggests that A-to-I editing of synaptic genes may be informative for assessing the epigenetic risk for autism, which is in line with the results of the current study and also a more recent study by Tran et al, in which they observed a global bias of hypoediting in a large cohort of postmortem brains of people with ASD (Tran et al., 2019). A global change of the editing profile is supposed to cause alterations in gene expression program associated with ASD. In the future study, it would be interesting to establish a direct link between purine metabolites and autistic genes.

DNA methylation has been shown to play regulatory roles in maintenance of genomic stability, defining tissue and cell type-specific gene expression, and regulation of cellular function in response to the environment and may provide valuable mechanistic insights into ASD (Ciernia and Lasalle, 2016). In the current study, a large excess of 2-Methylguanosine and N2,N2-Dimethylguanosine was observed in ASD, providing a clue for an altered DNA methylation in ASD. As 2-Methylguanosine was detected only in the female ASD subjects, sex-dependent and methylation modification-based regulatory mechanisms may exist. Indeed, retinoic acid-related orphan receptor alpha (RORA), a transcription factor involved in the sex steroid hormone expression regulatory pathway, has been identified to have elevated methylation in ASD and decreased expression in the ASD frontal cortex and cerebellum (Nguyen et al., 2010). Moreover, regulatory targets of RORA show sex-dependent differential expression levels (Hu et al., 2015; Sarachana et al., 2011) supporting a pattern of sex-dependent differential methylation in ASD. Previous studies also demonstrated that, the level of aromatase protein, a crucial enzyme in the biosynthesis of estrogen from testosterone, was strongly correlated with the level of the RORA protein in the brain tissues, and both RORA and aromatase

proteins were decreased in the frontal cortex of subjects with ASD. On the basis of these findings, it is speculated that a reduction in RORA observed in ASD may be exacerbated by a negative feedback mechanism involving decreased aromatase level, which in turn aggravates accumulation of testosterone and reduction in estradiol. Interestingly, we detected a significant increase in 7alpha-hydroxytestololactone only in the female samples. The structure of this compound is similar to the structure of testolactone, an aromatase inhibitor that blocks conversion of androgens to estrogens; the compound may have the same role as testolactone suppressing the activity of aromatase and leading to disruptive biosynthesis of estrogen from testosterone and reduction in RORA by a negative feedback mechanism reported in the previous studies. Alterations in the levels of 2-Methylguanosine and 7alpha-hydroxytestololactone in the urine of female subjects are likely linked to methylation regulation of RORA pathway and the interplay between RORA and aromatase, thereby being valuable for evaluating the epigenetic risk for autism, and also important for identification of the molecular pathways involved in ASD and male-biased biology.

Significant alterations in the arginine metabolism are observed as a reduction in urinary creatine and an increase in creatinine; in particular, this pattern is observed in the female subjects (Fig. 5). The endogenous synthesis of creatine requires two amino acids, glycine and arginine, and two enzymes, L-arginine:glycine amidinotransferase (AGAT), and N-guanidinoacetate methyltransferase (GAMT), along with a membrane transporter, encoded by the *SLC6A8* gene. Initially, AGAT catalyzes the transfer of the guanidino group from arginine to glycine to form ornithine and guanidinoacetate; this step occurs mainly in the kidney. The second step occurs essentially in the liver; GAMT catalyzes the transfer of a methyl group from S-adenosylmethionine to guanidinoacetate to form S-adenosylhomocysteine and creatine, which can enter the cells and tissues through a specific membrane transporter encoded by the *SLC6A8* gene. Thus, primary creatine deficiency disorders are directly caused by a deficiency in either AGAT, GAMT or X-linked recessive SLC6A8 creatine transporter; the disorder is known as cerebral creatine deficiency and has been reported in children with ASD (Aydin, 2018; Cameron et al., 2017; Longo et al., 2011; Pooarguelles et al., 2006). Creatine plays a relevant function in the cellular energy metabolism supplying high-energy phosphate groups to the cells through phosphorylation of creatine by creatine kinase. In our study, creatinine was significantly increased at the expense of high levels of creatine and phosphocreatine; thus, the cells will become rapidly depleted in energy, especially neurons, and reduced creatine content can explain neuronal injury observed in ASD. The influence of gender on creatine metabolism and transport has been proposed in the reports of the literature suggesting that inhibition by estrogens and stimulation by testosterone of SLC6A8 is a coherent basis for the higher leakage of body creatine to urine observed in the female subjects (Joncquel-Chevalier et al., 2013, 2015). In the current study, higher secretion of creatine (5,940,151 ± 3,933,993 vs 3,901,302 ± 2,638,589, peak intensity) and lower level of creatinine (6,221,110 ± 5,409,894 vs 24,429,825 ± 12,341,786) were observed in the urine samples of the female subjects compared with that in the male subjects in the control group. However, in the ASD group, an increase in 7alpha-hydroxytestololactone in the female subjects may reverse this pattern by inhibiting the conversion of testosterone to estrogen leading to accumulation of testosterone and reduction in estrogen, which exacerbates enhanced transport of creatine from the blood to the cells and finally leads to a reduction in creatine and an increase in creatinine in urine of the female ASD subjects. Nevertheless, these two metabolites are not significantly different between the male and female subjects with ASD.

Correlation analysis of these metabolites in the female subjects was conducted. As shown in Table 2, 7alpha-hydroxytestololactone was positively correlated with adenine (Pearson correlation coefficient, $r = 0.738$, $p < 0.01$), creatinine ($r = 0.826$, $p < 0.01$) and 2-Methylguanosine ($r = 0.757$, $p < 0.01$) and negatively correlated with creatine ($r = -0.413$, $p < 0.05$) in the female subjects. The results indicate that the effect of 7alpha-hydroxytestololactone on the hormones may play a key role in triggering the gender-related metabolic disturbances.

Ratios of metabolites can uncover biological properties that are not evident in the case of individual metabolites and increase the signal when two metabolites with a negative correlation are evaluated. For example, this strategy formed the basis of the standard phenylketonuria diagnostics using a ratio of phenylalanine and tyrosine (Eastman et al., 2000). Since creatinine is biosynthesized from creatine similar to phenylalanine and tyrosine and the former two metabolites are very commonly used in routine testing, we evaluated the ratio of creatinine to creatine as a predictor of ASD diagnosis. As shown in Fig. 6A, in the female subjects, a receiver operating characteristic (ROC) curve analysis using the creatinine:creatine ratio yields an area under the curve (AUC) of 0.913 (95% CI: 0.806–1). Scatter plots of the creatinine:creatine ratio were used to create a diagnostic test (Fig. 6B); the red horizontal line is the diagnostic threshold (threshold = 2.71), which clearly distinguishes ASD from the control with a specificity of 0.824 and a high sensitivity of 1.000. However, in the male subjects, a poor AUC value of 0.511 was obtained and it is difficult to distinguish

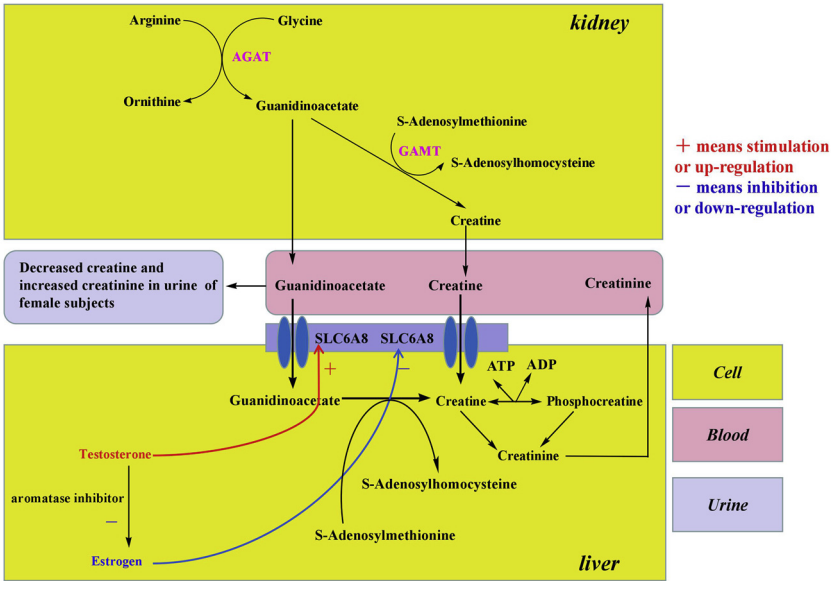

Fig. 5. Creatine synthesis and transport. The mechanism involved of sex hormones is in part currently hypothetic and might explain unexpected decrease of creatine and increase of creatinine in female ASD subjects. AGAT: L-arginine:glycine amidinotransferase, GAMT: N-guanidinoacetate methyltransferase, ATP: adenosine triphosphate, ADP: adenosine diphosphate.

Table 2
Correlation analyses among those metabolites observed in female subjects.

	Creatine	Creatinine	7alpha-hydroxy-testololactone	Adenine	2-Methylguanosine
Creatine		r = -0.277 p = 0.112	r = -0.413 p = 0.015	r = -0.195 p = 0.268	r = -0.207 p = 0.240
Creatinine	r = -0.277 p = 0.112		r = 0.826 p < 0.01	r = 0.784 p < 0.01	r = 0.775 p < 0.01
7alpha-hydroxytestololactone	r = -0.413 p = 0.015	r = 0.826 p < 0.01		r = 0.738 p < 0.01	r = 0.757 p < 0.01
Adenine	r = -0.195 p = 0.268	r = 0.784 p < 0.01	r = 0.738 p < 0.01		r = 0.897 p < 0.01
2-Methylguanosine	r = -0.207 p = 0.240	r = 0.775 p < 0.01	r = 0.757 p < 0.01	r = 0.897 p < 0.01	

[r]Pearson correlation coefficient.

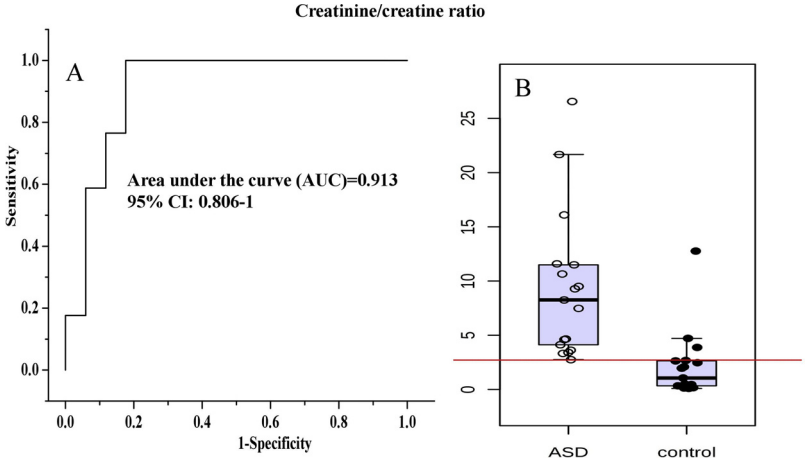

Fig. 6. ROC curve and scatter plots of creatinine:creatine ratio in female subjects. The red horizontal line is the diagnostic threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

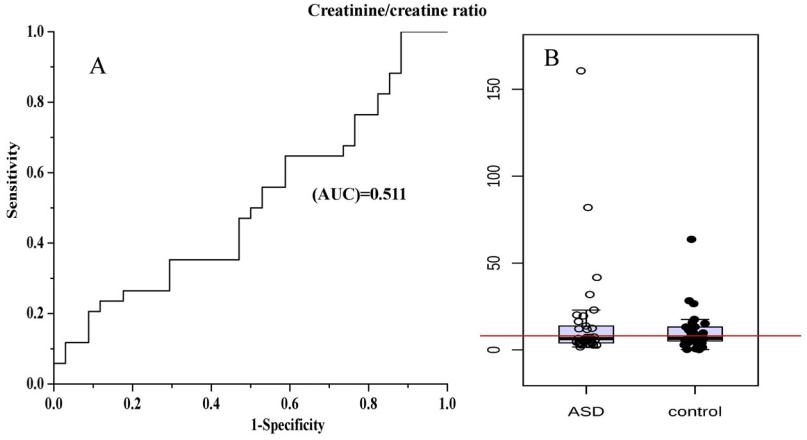

Fig. 7. ROC curve and scatter plots of creatinine:creatine ratio in male subjects. The red horizontal line is the diagnostic threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between ASD and the control (Fig. 7). These results show that the creatinine to creatine ratio may be a good predictor of ASD in the female subjects.

5. Conclusions

A comprehensive metabolomics study was performed to search for gender-related metabolites in autism spectrum disorders using the sensitive HPLC-QTOF-MS approach in the positive and negative ESI modes. No discriminating metabolites between the male and female subjects with ASD were identified; however, changes in 5 sex-sensitive metabolites were observed in the female subjects. Interestingly, 7alpha-hydroxytestololactone was positively correlated with adenine, creatinine and 2-Methylguanosine and negatively correlated with creatine possibly suggesting an association with the sex hormone pathway. These results may provide very interesting leads toward possible pathophysiological explanations for the gender bias in manifestation of ASD. A high AUC value obtained by ROC analysis revealed that the creatinine to creatine ratio may have a potential use as a biomarker for the clinical diagnosis and evaluation of ASD in the female subjects. Further investigations with a larger group of ASD patients (especially involving recruitment of sufficient number of the female patients) will

be necessary in the near future to verify an extent of the gender bias-based reflection of intrinsic metabolic variations.

Author contributions

X.Y.X., X.Q.S., T.Y.L., and Y.C.W. designed the study; D.L., W.J.H., W.S.Z. collected the data; D.H.X., H.L., T.Z. performed the experiments; X.Y.X., Y.C.W. analyzed the results, X.Y.X. wrote the main manuscript text. X.Y.X., X.Q.S., T.Y.L., and Y.C.W. reviewed the manuscript.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105594.

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