Alterations in Gut Vitamins and Amino Acids Metabolism are Associated with Symptoms and Neurodevelopment of Children with Autism Spectrum Disorders

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Research

Keywords: autism, metabolomics, metabolism, vitamin, symptoms, children

Posted Date: August 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-59819/v1

Version of Record: A version of this preprint was published at Journal of Autism and Developmental Disorders on July 14th, 2021. See the published version at https://doi.org/10.1007/s10803-021-05066-w.

Abstract

Background Accumulated evidence have supported metabolic disturbance may be associated with the pathogenesis of autism spectrum disorders (ASD). Despite abnormalities of some shared metabolic pathways, specific differential compounds are inconsistent in studies, which made a challenge to elucidate the role of metabolism in the mechanism of ASD. Besides, few researches have assessed the correlation between gut metabolites with symptoms of ASD.

Objectives The present study aimed to evaluate the gut metabolomic profiles of children with ASD and to analyze potential interaction between gut metabolites with symptoms and neurodevelopment of ASD children.

Methods In this cross-sectional case-control study, 120 aged 2–6 years ASD children and 60 sex and age matched typically developing (TD) children were included. Autistic symptoms were assessed with the Autism Behavior Checklist (ABC), Childhood Autism Rating Scale (CARS), and the Social Responsiveness Scale (SRS). Neurodevelopment was assessed with the Gesell Developmental Scale (GDS). Fecal samples were analyzed by untargeted liquid chromatography-mass spectrometry (LC-MS) methods, then systematic bioinformatic analyses were performed to characterize the gut metabolomic profiles of ASD and TD children. The correlations between metabolites and clinical assessment scores were assessed using Spearman correlation.

Results ASD children exhibit gut metabolism perturbation compared with TD children. A total of 96 differential metabolites between the ASD and TD groups were identified, with 35 increased and 61 decreased in ASD group. The metabolic disturbance of ASD involved in multiple vitamins and amino acids metabolism pathways, with the strongest enrichment identified for tryptophan metabolism, retinol metabolism, cysteine and methionine metabolism, and vitamin digestion and absorption. The imbalanced gut metabolites are significantly correlated to symptoms and neurodevelopment of ASD children.

Limitations This cross-sectional study revealed a correlation, but do not allow to prove causation of symptoms and gut metabolites outcome. The disease specificity of the metabolomic disturbance need to be evaluated in future studies.

Conclusions ASD children have altered gut metabolite profiles compared with TD children, which mainly involved in multiple vitamins and amino acids metabolism pathways. Notably, vitamins metabolism abnormalities may play roles in the disturbance of amino acids metabolism. Imbalanced gut metabolites are related to symptoms and neurodevelopment of ASD children. Our findings provided an improved understanding of perturbations of metabolome networks in ASD.

1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by early-appearing social communication deficits and restricted or repetitive behaviors[1]. Besides the core deficits, ASD are often accompanied with other developmental or behavioral disorders, sleep problems, and gastrointestinal (GI) symptoms[1]. Currently, the exact etiology and mechanism of ASD are mostly unclear, thus hindering the development of available laboratory diagnostic and effective cure for the condition[2]. Accumulated evidence has supported metabolic disturbance may be associated with the pathogenesis of ASD. Metabolomics studies based on urines, plasma, and fecal samples of ASD patients showed some common metabolic disturbances, including some amino acids metabolites, oxidative stress, purine intermediates, and gut microbiota metabolism[3]–[6].

Despite the high interest, previous studies are mainly focused on the metabolomic analysis of urines and blood, and analysis of gut microbiota composition, while studies of fecal metabolism are relatively rare in the context of ASD[4][6]. Gut metabolomics have the advantage to provide comprehensive information of the final products of nutrients intake, metabolism, and microbial functions. Studies have shown that short-chain fatty acids (SCFAs) altered in the gut of ASD children [7][8],and SCFAs can regulate gut immune and genes expression of the host[9]. Abnormalities of glutamate and GABA metabolism were also observed in the feces of ASD children, which may influence the balance between excitation and inhibition[6][10]. Abnormality of tryptophan metabolism and increase of serotonin (5-hydroxytryptamine, 5-HT) in gut of ASD patients has been reported in several studies[11]. Also, isopropanol and phenol substances, including phenol and *p*-cresol, were found higher in fecal of children with ASD[6][12]. These findings suggest that alterations of gut metabolomics may play an important role in the pathogenesis of ASD.

However, specific compounds are inconsistencies between studies, for multiple potential confounds including ethnicity, age, diets, disease, and medicine can influence the metabolism status. Besides, methodology of is also a critical factor impact the metabolism findings. Inconsistent and scattered changes of single metabolites have a limited role in elucidating the pathophysiology of ASD, and thus a comprehensive interpretation of the metabolism pathway network may facilitate exploring the pathogenesis of ASD.

In the present study, we analyzed fecal metabolomic profiles of preschool children with ASD and age, sex, region matched typically developing (TD) children by liquid chromatography-mass spectrometry (LC -MS) methods. We found the differential metabolites between ASD and TD children mainly involved in multiple vitamins and amino acids metabolism pathways. We also investigated the possible link between the gut metabolites with symptoms and neurodevelopment of ASD children, and postulated the interconnection of vitamins and amino acids in the metabolism network of ASD.

2. Subjects And Methods

2.1. Subjects

A total of 120 ASD children aged 2-6 years were selected for this study from the Maternal and Child Care Health Hospital of Hainan Province, China, after a comprehensive assessment. Inclusion criteria were a diagnosis of ASD, which was made by developmental pediatrician of our research team through a series of structured interviews according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria[13]. The Childhood Autism Rating Scale (CARS)[14] was used as an assistant to diagnose by scores above the cut-off point 30. Exclusion criteria included a history of other developmental disorders, neurological or psychiatric diseases, genetic metabolic disease, major physical illness, recent infection, recent use of special diets, recent use of antibiotics or probiotics within one months before sampling.

Symptoms of the children with ASD were assessed with the Autism Behavior Checklist (ABC)[14], Social Responsiveness Scale (SRS) [15]and the CARS test. Higher scores of ABC, CARS, and SRS scores indicate more serious autistic symptoms. Neurodevelopment in ASD children was assessed with the revised Gesell Developmental Scale (GDS) [16]which is extensively used in China to evaluate cognitional and behavioral development, and the development quotient scores (DQ) reflect the levels of intellectual and behavioral development. DQ <75 indicates developmental delay, and the lower DQ score, the more severe developmental delay.

Additionally, a control group of 60 typically developing (TD) children was recruited and matched to the ASD group by age, gender, and region. The TD children received health examinations at the Department of Child Health in Maternal and Child Care Health Hospital of Hainan Province. They are healthy, and they did not have any signs of developmental disorders or psychiatric diseases, and noticeable gastrointestinal symptoms. Other exclusion criteria were the same as for the ASD group.

Participation in this research was voluntary. The study protocol was approved by the institutional review board of Children's Hospital, Chongqing Medical University. This cross-sectional case-control study was based on a clinical trial which was registered in the Chinese Clinical Trial Registry (ChiCTR; registration number: ChiCTR-ROC-14005442).

2.2. Fecal sample collection and LC-MS metabolomics analysis

2.2.1. Fecal sample collection

Fecal samples were collected from each participant and immediately frozen and stored at −80°C until further analysis. The 100 mg of stool for each sample was preserved in sterile tubes for metabolism analysis.

2.2.2.Metabolites extraction

The 100 mg of stool for each sample was separately ground with liquid nitrogen and the homogenate was resuspended in prechilled 80% methanol and 0.1% formic acid by vortexing thoroughly. Samples were incubated on ice for 5 min then centrifuged at 15,000 rpm at 4°C for 5 min. Some supernatants were diluted with LC-MS-grade water to a final concentration of 60% methanol. Hereafter, samples were

transferred into a fresh Eppendorf tube through a 0.22 μm filter then centrifuged at 15,000 g at 4°C for 10 min. Finally, the filtrate was injected into the LC-MS/MS system for analysis.

2.2.3. UHPLC-MS/MS analysis

LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher, USA) and an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher, USA). Briefly, metabolites were first separated and characterized by using a liquid chromatography system and further detected with a mass spectrometry system. Samples were injected onto an Hyperil Gold column (100 × 2.1 mm, 1.9 μm) at a flow rate of 0.2 mL/min and separated using a 16 min linear gradient. The eluents for positive polarity mode were eluent A (0.1% formic acid in water) and eluent B (methanol). The eluents for negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (methanol). The solvent gradient was set as follows: 2% B 1.5 min, 2-100% B 12.0 min, 100% B 14.0 min, 100-2% B 14.1 min, 2% B 16 min. The Q Exactive HF-X mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.2 kV, a sheath gas flow rate of 35 arb, an aux gas flow rate of 10 arb, and capillary temperature of 320°C.

2.2.4 Metabolite analysis

Compound Discoverer 3.0 (CD 3.0, Thermo Fisher) was used to process and normalize the raw data files generated by UHPLC-MS/MS to perform peak alignment, peak selection, and quantification for each metabolite. The main parameters were set as follows: retention time tolerance 0.2 min, actual mass tolerance 5 ppm, signal intensity tolerance 30%, signal/noise ratio 3, minimum intensity 100,000. Peak intensities were normalized against the total spectral intensity, and normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. Peaks were matched with mzCloud (https://www.mzcloud.org/) and ChemSpider (http://www.chemspider.com/) databases to obtain accurate qualitative and relative quantitative results.

The normalized metabolism data were analyzed by the CentOS (CentOS release 6.6), statistical software R (R version R-3.4.3), and SPSS statistical software (version 19.0, SPSS Inc., USA). With individual metabolite dataset, Partial least squares discriminant analysis (PLS-DA) models were built to visualize the metabolic alteration patterns between ASD and TD children. Furthermore, the cross-validation ANOVA (CV-ANOVA) was calculated to assess the reliability of the models. Differential metabolites between the two groups were selected by combined multivariate and univariate analysis methods. Gut metabolites with fold change >1.5, variable important in projection (VIP) >1, and FDR-corrected P values < 0.05 for student's t-test or Mann-Whitney U test were considered significantly differential metabolites between groups. To further demonstrate the biological functions of the associated differential metabolites, the KEGG pathways enrichment analysis was performed (http://www.genome.jp/kegg/), and a hypergeometric test was used to assess the significance of KEGG pathway.

The metabolomics analysis was carried according to the standard protocols recommended by Novogene Technology Co., Ltd. (Beijing, China). The raw data were deposited into the MetaboLights database

(accession number: MTBLS1946, www.ebi.ac.uk/metabolights).

2.3. Statistical Analysis

The demographics and clinical assessment data were analyzed using SPSS statistical software (version 19.0, SPSS Inc., USA). Continuous variables are described as the means with standard deviations or medians (interquartile ranges) when appropriate, and categorical variables are described as percentages. The two-tailed student's t-test, Mann-Whitney U test, and the chi-square test were used to compare levels between groups. The correlations between metabolites levels with clinical symptoms scores were analyzed by Spearman correlation. *P*-value < 0.05 was presumed as statistically significance.

3. Results

3.1 Characteristics of the subjects

A total of 120 ASD children aged 2-6 years and 60 TD children matched to the ASD group by age, gender and region were selected for this study. Demographic information and clinical features were shown in Table 1. There were no significant differences in age-gender composition and z-score of body mass index (BMI) between the two groups. In all of 120 ASD children, 80(66.67%) showed food selectivity, and 58(48.33%) had GI symptoms.

3.2. Alterations in gut metabolism profiles of ASD children

To explore the gut metabolic patterns associated with ASD status, a fecal metabolome analysis was performed by LC-MS/MS. A total of 6936 peaks of compounds were obtained, among which 4531 were explored in positive ion mode (ESI+) and 2405 in negative ion mode (ESI-). The supervised PLS-DA showed that the ASD and TD groups were well-clustered with particular metabolic profiles for each(ESI+: R2Y = 0.73，Q2 = 0.61, *P* <0.001 ; ESI-: R2Y = 0.76，Q2 = 0.65, *P* <0.001) (Figure 1a-b). The permutation test with *P* <0.001 indicates that the classification of global metabolite profiles between ASD and TD are significantly different. A total of 96 differential metabolites between the ASD and TD groups were identified, 35 of which were significantly increased in the ASD group, and 61 metabolites were decreased in the ASD group. Table S1 showed the list of the differential gut metabolites between the ASD and TD groups that achieved statistical significance.

To further demonstrate biological functions of the differential metabolites, KEGG pathways enrichment analysis was performed. Twenty-seven KEGG pathways were associated with ASD status (Table S2). Interestingly, the differential metabolites mainly participated in multiple vitamins and amino acids metabolism pathway, with the strongest enrichment identified for tryptophan metabolism (*P* = 0.0006), retinol metabolism(*P* = 0.009), and cysteine and methionine metabolism (*P* = 0.008), and vitamin digestion and absorption (*P* = 0.01) (Figure 1c). Also, some differential metabolites involved in arachidonic acid, steroid hormone, citrate cycle, and purine metabolism.

As shown in Table 2 and Figure 2, disturbances of various vitamins metabolism were found in children with ASD. In the retinol metabolism pathway, precursors and intermediates of vitamin A showed abnormal levels in fecal of ASD children. Level of 4'-apo-beta-carotenal, b,e-carotene-3,3'-diol, and retinal were increased while retinol was decreased. Concentrations of multiple vitamins B and their derivates were decreased, including thiamine-pyrophosphate (TPP), riboflavin (vitamin B2) and intermediates, vitamin B5, vitamin B6, nicotinate, dihydrofolate (DHF), and 5-methyltetrahydrofolate(5-MTHF). Vitamin C level was also decreased in the feces of children with ASD.

Aberrant amino acids metabolisms were associated with ASD status (Figure 2). In the tryptophan metabolism pathway, concentrations of xanthurenic acid, 5-hydroxy-N-formylkynurenine, 5-hydroxytryptophan (5-HTP), serotonin (5-hydroxytryptamine, 5-HT) and N-feruloyl serotonin were significantly increased in feces of children with ASD. In contrast, 6-hydroxymelatonin and 5-hydroxyindoleacetic acid (5-HIAA) were lower in ASD children. The cysteine-methionine metabolism pathway is closely relevant to folate metabolism. Both pathways were abnormality presents with a lower level of DHF, 5-MTHF, carnitine N-acetylcysteine (NAC), and S-aminoethyl- cysteine, while an excessive accumulation of homocysteine (Hcy) in ASD children. At arginine metabolism, concentrations of polyamines, including agmatine, spermine, and glutathione spermidine, were lower in ASD children. We also find abnormal glutamate metabolism and glycine metabolism, with decreased glutamine, γ-aminobutyric acid (GABA), and glycine in children with ASD.

Biologically active metabolites of arachidonic acid showed disturbance, which were crucial regulators in oxidative stress and inflammation. Arachidic acid and 20-hydroxy-leukotriene E4 were increased while leukotriene B4 and 5-trans prostaglandin F2β were decreased in feces of ASD children. Besides, 8-hydroxy-deoxyguanosine(8-OHdG), a purine metabolite, is a sensitive marker of oxidative DNA damage[17]. In the present study, 8-OHdG was significantly increased with 6.86-fold (P = 0.001) in autistic children compared to TD children.

3.3. Correlation between gut metabolites with symptoms and neurodevelopment of ASD children

Spearman correlation were performed to explore the potential links between key fecal metabolites and clinical assessment scores of ASD children (Figure 3 showed the significant correlations). The fecal concentrations of S-aminoethyl-L-cysteine, 5-trans prostaglandin F2β□retinol, and riboflavin were positively correlated with neurodevelopment scores, while 8-OHdG, 5-hydroxy-N-formylkynurenine, Hcy, retinal, and serotonin were negatively correlated with neurodevelopment scores. The levels of agmatine, S-aminoethyl-L-cysteine, pyridoxamine, GABA, and 5-trans prostaglandin F2β were negatively correlated with partial subscales or total ABC, SRS or CARS scores. Conversely, concentrations of retinal, Hcy, serotonin, N-feruloyl serotonin, and 5-HIAA in the gut were positively correlated with symptoms of ASD children.

4. Discussion

This study reports significantly different gut metabolomic profiles between young children with ASD and TD children. Interestingly, the differential fecal metabolites are majorly involved in multiple vitamins and amino acids metabolism pathways, with the strongest enrichment identified for tryptophan metabolism, retinol metabolism, cysteine and methionine metabolism, and vitamin digestion and absorption. Some of the metabolic perturbations were associated with symptoms and neurodevelopment of ASD children, which may play important roles in the pathogenesis of ASD through the “gut-brain axis”.

Vitamin A is an important micronutrient for the systemic development and function of children[18]. Vitamin A deficiency (VAD) is still a public health issue in many developing countries. Studies showed ASD children are more vulnerable to VAD than neurotypical children[19][20]. In our study, an increased level of 4'-apo-beta-carotenal, b,e-carotene-3,3'-diol and decreased retinol may indicate that ASD children had decreased capacity of absorption and bioconversion of plant-origin precursors of vitamin A. Vitamin A has three active forms, retinal, retinol and retinoic acid (RA) in humans[21]. RA, the main active form of vitamin A, is a crucial signaling molecule that regulate multiple fundamental biological processes[21]. Increased retinal in our study may imply it was suppressed to convert to RA in the gut of ASD children, and excessive retinal may damage the nervous system. We found retinol level was positively correlated with neurodevelopment level while retinal was positively related to the social withdrawal of SRS in ASD children. ALDH1A family are key enzymes to oxidize retinal into RA, and XX Xu et al.[22] found ASD patients with excessive UBE3A (an autism related gene and molecule) may have congenital errors of retinol metabolism, for excessive UBE3A can inhibit the activity of ALDH1A and compromised retinal oxidized to RA. Moreover, gut microbiota can take part in alternative biotransformation of retinal to retinol or RA[23].

Vitamins B are important cofactors implicated in multiple biochemical reactions. TPP, a derivative of thiamine (vitamin B1), is a cofactor of various enzymes in the mitochondria 5. Anwar A et al.[24] found TPP concentration in the plasma of ASD children was significantly reduced compared to controls. We also found a lower level of TPP in feces of children with ASD. Decreased TPP can lead to a reduced potential of anti-oxidative and energy produce in mitochondria, and subsequently cellular[25] [26]. Vitamin B2 and B6 also participate in multiple amino acids metabolism process. We also found the level of pyridoxamine, a form of vitamin B6, was slightly negatively related to the ABC and CARS scores of ASD children.

The pathway of cysteine and methionine cycle, folate(vitamin B9) metabolism, and Hcy transsulfuration are interrelated and constitute the folate-related metabolism together[27]. The folate-related pathway has a critical role in cell proliferation, DNA synthesis, immune function, and neural development[28]. Vitamin B6 and B12 are cofactors of these biological processes. Decreased folate and vitamin B6 may lead to an accumulation of homocysteine and decreased methyl production. Much of evidence suggested that folate deficit and excessive Hcy are risk factors of neural tube defects and neurodevelopmental disorders [29]. Several studies showed that children with ASD had decreased folate and elevated levels of Hcy in blood and urine [30][31]. There was a negative correlation between Hcy levels with neurodevelopment scores in our study, supporting the adverse impact of excessive Hcy on brain development and function.

Moreover, NAC is an antioxidant, and clinical trials showed NAC has potential benefit in treating the irritability of ASD children[32].

Abnormality of tryptophan metabolism in patients with ASD has been reported in multiple studies, which was characterized by decreased concentrations of tryptophan[33] and increased levels of serotonin in blood [11]. In the gut, there are three main tryptophan metabolism pathways leading to serotonin, kynurenine, and indole derivatives[34][35]. Through the kynurenine pathway, kynurenic acid, xanthurenic acid, and quinolinic acid are generated[35]. In our study, concentrations of xanthurenic acid and 5-hydroxy-N-formylkynurenine were significantly increased in the feces of children with ASD. Vitamin B6 is a cofactor of kynureninase and kynurenine aminotransferase; therefore, the decrease of B6 may contribute to the increased levels of xanthurenic acid and 5-hydroxy-N-formylkynurenine. In the serotonin pathway, 5-HTP, serotonin, and N-feruloyl serotonin were significantly increased in feces of children with ASD, while 6-hydroxymelatonin and 5-HIAA were lower in ASD children. Reproducible evidence suggested impaired serotonin-melatonin pathway in ASD characterized by hyperserotonemia and melatonin deficit in plasma [11][36][37]. However, few studies reported tryptophan metabolism and serotonin- melatonin levels in the gut of ASD patients. MD Angelis et al. [12] found higher amounts of tryptophan and 3-methylindole in the feces of children with ASD. Dan Z et al. [38] also reported abnormal metabolism of tryptophan in the gut of ASD children. An experiment in mice model of autism found a decrease of serotonin in intestine mucosal[39]. However, given 95% of the serotonin in body is generated in the intestine[40], it is likely that blood serotonin levels are correlated with enteric serotonin. Likewise, gastrointestinal tract are also important source of melatonin besides the pineal gland[41]. Melatonin can regulate sleep patterns, immune system, as well as gastrointestinal function[41]. Serotonin can be catabolized to 5-HIAA, and this process depends on riboflavin (vitamin B2) as a cofactor, so riboflavin deficiency may be related to the increase of serotonin. Moreover, dysbiosis of gut microbiota was linked to abnormal tryptophan metabolism[35]. We found a correlation between gut serotonin levels with neurodevelopment scores of ASD children, while serotonin and N-feruloyl serotonin levels were positively correlated with sensory subscales of ABC. Many researches have indicated that the blood levels of serotonin are correlated with autism severity[36]. A Balanced amount of enteric serotonin is beneficial to the functioning of the intestine, nervous system, and gut-brain axis, while excess serotonin may play a harmful role in the progression of ASD.

We found decreased GABA, glutamine, glycine, and polyamines in fecal of children with ASD. GABA were negatively correlated with ABC scores, and agmatine were negatively correlated with SRS scores. These amino derivates are crucial neurotransmitters or neuromodulators in nerve system, and yet are important media of immune and inflammation[42]. GABA and glycine are inhibitory neurotransmitters, and their decrease may impact the excitation-inhibition balance of nervous system [43]. Our findings were partial supported by previous study of Kang DW et al[6]and Angelis et al.[12], which showed possibly lower GABA concentrations in guts of children with ASD compared with healthy controls. Ford et al. found aberrant glutamate and GABA processes are linked with impaired psychosocial function[44]. Particularly, the synthesis of GABA and glycine depend on vitamin B6 as cofactor[45].

Biologically active metabolites of arachidonic acid showed disturbance, which were key regulators in oxidative stress and inflammation[46]. Besides, 8-OHdG, a purine metabolite, is a sensitive marker of oxidative DNA damage[17]. Studies showed evaluated 8-OHdG levels in cerebellar[47] and urinary excretion[48] of ASD patients. In the present study, 8-OHdG was significantly increased with 6.86-fold in autistic children compared to TD children. These results in together indicate that the gastrointestinal tract of ASD children may have higher risk damaged by oxidative stress and inflammation.

Gut microbiota was an important role in the gut metabolism, for microflora can produce vitamins and participant in the metabolic of numerous substances [49][23]. Inadequate intake from food could also partly explain the decreased of multiple vitamins and amino acids. Further, the abnormality and deficiency of vitamins may play roles in the disturbance of amino acids metabolism, for vitamins B are implicated in multiple biochemical reactions[45]. Metabolic interventions for ASD patients mainly include supplementation of prebiotics and probiotics, vitamins (e.g., A, C, D, B6, B12, folate), amino acids and derivates (e.g., glycine, N-acetylcysteine)[50]–[53]. Approaches above could sometimes correct the dysbiosis of intestinal flora or nutritional deficiencies in ASD, and partly improve the downstream metabolic consequences. However, these interventions were not always effective[53], for some inborn metabolism errors are hard to be rectified, and single compound supplementation may be insufficient for extensive abnormalities of metabolic networks in ASD. Besides, metabolism anomaly was only one of many factors related to neurological function and symptoms of ASD.

Limitations

There are limitations in the present study. This cross-sectional study revealed a correlation, but our data do not allow to prove causation of symptoms and gut metabolites outcome. And the correlations were not very strong (correlation coefficient 0.2–0.4), for the metabolic disturbance was just one of many factors related to neurological function and symptoms of ASD. It is unlikely to distinguish whether the metabolites are derived from the host or the gut microbiota. Our participants were preschool children from an island of China with a comparable biology backgrounds; the findings may not be generalizable to all ASD patients in different regions, races, and ages. ASD children were mostly accompanied by other developmental or behavioral disorders. In our study, 81.7% ASD children had developmental delay, so studies involving different ASD subtypes and other related diseases are needed to evaluate the disease specificity of the metabolomic disturbance. Some metabolic disturbances may be nonspecific for various neurodevelopmental diseases and have an extensive impact on brain function and neurodevelopment.

Conclusions

In conclusion, ASD children exhibit gut metabolism perturbation mainly associated amino acids and vitamins metabolism, and the imbalance of gut metabolism are related to symptoms and neurodevelopment of children with ASD. Aberrant of gut metabolism profiles may be the result of the interaction of multiple factors, including congenital metabolism errors, decreased intake by abnormal eating pattern, and intestinal microflora imbalance (Fig. 4). Notably, in the interrelated metabolism

networks, vitamins metabolism abnormalities and decreased intake of vitamins may disturb the amino acids metabolism, for vitamins B are essential cofactors implicated in multiple biochemical reactions. The metabolites may affect the brain development and function, and subsequently behavior by nutrients, neurotransmitter, immune-inflammation modulatory, and other pathways. Approach of nutritional supplements and regulating the intestinal flora may be partially beneficial to gut metabolism, nutritional status, and symptoms of ASD. It is essential to formulate detailed evaluation and provide comprehensive and individualized interventions for ASD children. Our findings provided an extensive understanding of the disturbances of metabolism networks in ASD.

Abbreviations

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: 5-hydroxytryptamine

5-HTP: 5-hydroxytryptophan

5-MTHF: 5-methyltetrahydrofolate

8-OHdG: 8-hydroxy-deoxyguanosine

ABC: Autism Behavior Checklist

ASD: autism spectrum disorder

BMI: body mass index

CARS: Childhood Autism Rating Scale

DHF: dihydrofolate

DQ: Developmental Quotient

GABA: γ-aminobutyric acid

GDS: Gesell Developmental Scale

GI: gastrointestinal

Hcy: homocysteine

LC-MS: liquid chromatography-mass spectrometry

NAC: N-acetylcysteine

PLS-DA: Partial least squares discriminant analysis

SRS: Social Responsiveness Scale

TD: typically developing

TPP: thiamine-pyrophosphate

VAD: vitamin A deficiency

VIP: variable important in projection

Declarations

Ethics approval and consent to participate

Participation in this research was voluntary. Parents signed written informed consent forms and were willing to let their children participate in the study. The study protocol was approved by the institutional review board of Children's Hospital, Chongqing Medical University. This cross-sectional case-control study was based on a clinical trial which was registered in the Chinese Clinical Trial Registry (ChiCTR; registration number: ChiCTR-ROC-14005442).

Consent for publication

Not applicable.

Availability of data and materials

All data generated and analyzed in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study is funded by the National Natural Science Foundation of China (No. 81372950) (http://www.nsfc.gov.cn) and Guangdong Province (2018B030335001) and Guangzhou City (202007030002) Key Project.

Authors' contributions

J Zhu, J Chen, and TY Li were involved in designing the trial and writing the trial protocol, analyzing the data and writing the manuscript. J Chen, L Li and TY Li were also involved in clinical assessment, supervising subjects' recruitment, data collection and drafting the manuscript. J Zhu, XY Hua, T Yang, M Guo, Q Li, and L Xiao were involved in experimental operation, data collection, analyzing the data and

revising the manuscript. J Chen, and TY Li had primary responsibility for final content. All authors approved the final version of the manuscript.

Acknowledgements

Not applicable.

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Tables

Table 1. Demographic and clinical characteristics of participants

	TD	ASD	*P* value
Age（years）（mean ±SD	4.01±1.12	3.86±1.03	0.2182
Sex（male/female）	39/21	99/21	0.079
Family annual income per capita (RMB), n (%)			
<=20000	31（51.67）	67（55.83）	0.597
>20000	29（48.33）	53（44.17）	
Height (ZHA)	0.01 ± 0.94	-0.14 ±1.0	0.2429
Weight (ZWA)	-0.02 ± 0.98	0.06 ± 0.96	0.5771
BMI (ZBMI)	0.26 ± 0.92	0.33 ± 1.07	0.6646
Picky eating, n (%)	26（43.33）	80（66.67）	0.003
GI symptoms, n (%)	0	58(48.33)	
ABC			
Sensory	-	8.37 ± 5.04	
Social withdrawal	-	14.13 ± 7.74	
Stereotypic behavior	-	7.63 ± 7.01	
Inappropriate speech	-	13.57 ± 5.98	
Laggard daily living ability	-	11.28 ± 5.18	
Total ABC scores	-	54.98 ± 22.74	
SRS			
Social awareness	-	11.83 ± 3.09	
Social cognition	-	18.68 ± 4.52	
Social communication	-	33.29 ± 8.82	
Social motivation	-	14.96 ± 4.4	
Autistic mannerisms	-	13.71 ± 5.58	
Total SRS scores	-	92.47 ± 21.65	
CARS	-	37.18 ± 5.86	
GDS	-		

Adaptive behavior	-	57.01±17.94
Gross motor	-	64±14.22
Fine motor	-	57.45±17.05
Language	-	43.62±19.97
Personal-social behavior	-	48.47±15.24

The two-tailed Student's t test, and the chi-square test were used to analysis. TD = typically developing; ASD = autism spectrum disorders.

Table 2. Aberrant in gut metabolites relevant to vitamins and cofactors in ASD children

Vitamins metabolites	Metabolism pathway	Fold change [a]	P value	Regulate mode [b]
4'-apo-beta-carotenal	Vitamin digestion and absorption, retinol metabolism	1.55	9.24E-05	up
b,e-Carotene-3,3'-diol	Vitamin digestion and absorption, retinol metabolism	1.56	0.0043	up
All-trans-retinal	Retinol metabolism	1.56	0.0262	up
Retinol	Retinol metabolism	0.64	0.0149	down
Tocopherol	Vitamin E metabolism	3.19	0.002	up
Thiamine pyrophosphate	Thiamine metabolism	0.45	0.0224	down
Riboflavin Tetrabutyrate	Riboflavin metabolism	0.21	0.0085	down
(+)-Riboflavin	Riboflavin metabolism	0.62	0.0305	down
Lumichrome	Riboflavin metabolism	0.64	0.005	down
Pyridoxamine	Vitamin digestion and absorption, vitamin B6 metabolism	0.63	0.0035	down
Phosphopantothenic acid	Vitamin B5 metabolism	0.64	0.0181	down
5-Methyltetrahydrofolate	Folate biosynthesis	0.56	0.0068	down
Dihydrofolic acid	Vitamin digestion and absorption, folate biosynthesis	0.49	0.006	down
1,4,5,6-tetrahydro-6-oxonicotinic acid	Nicotinate and nicotinamide metabolism	0.66	0.0036	down
Vitamin C	Vitamin digestion and absorption, ascorbate and aldarate metabolism	0.58	0.0148	down
L-Ascorbic acid	Ascorbate and aldarate metabolism	0.51	0.0031	down

[ab] Fold change and regulate mode of ASD group compared with typically developing group. ASD = autism spectrum disorders.

Figures

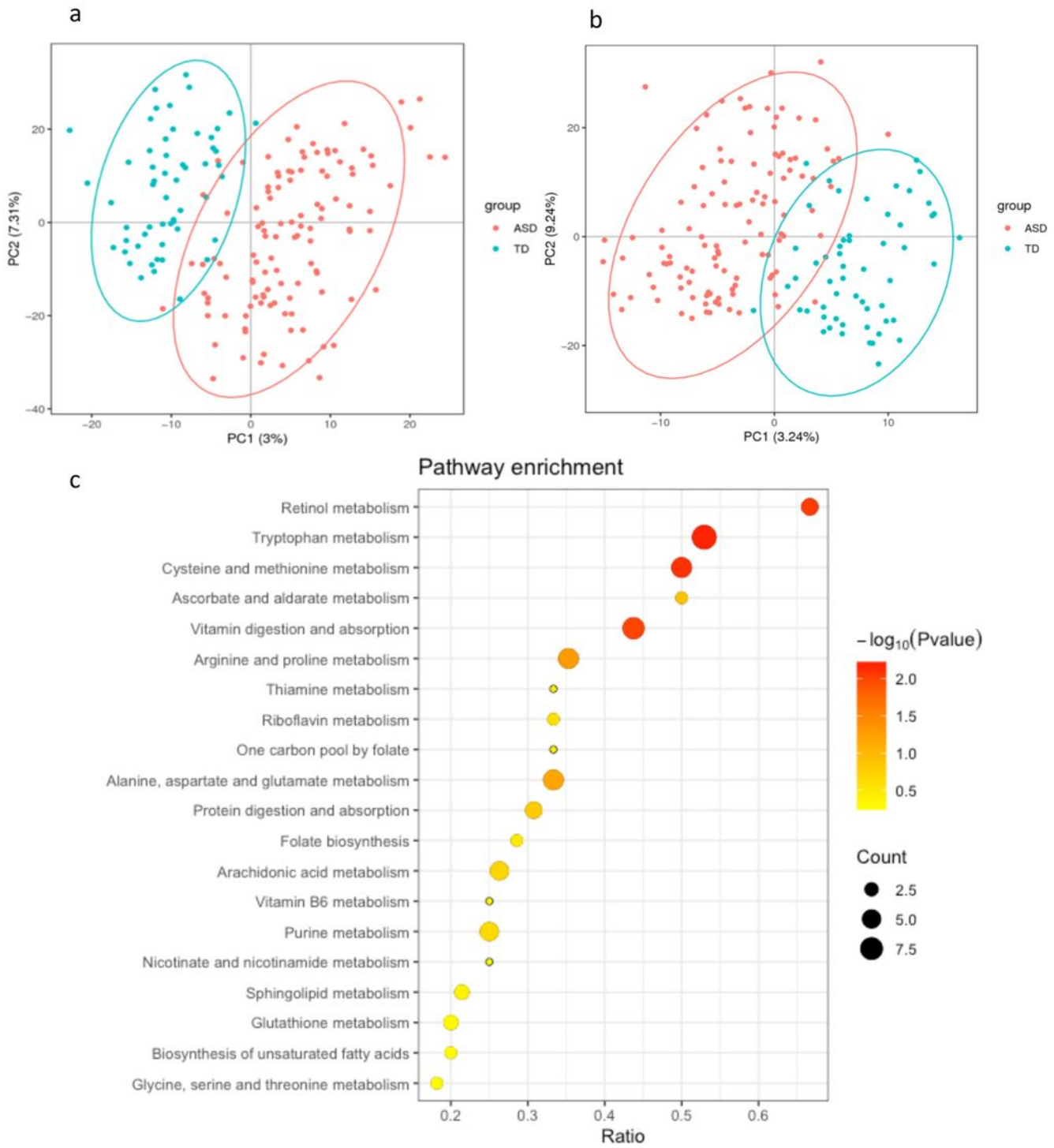

Figure 1

Alterations in the gut metabolome of children with ASD compared with typically developing children. (a, b) The clustering analyses of partial least-squares discriminant analysis (PLS-DA) of gut metabolome data in positive ion mode (a) and negative ion mode (b). (c) Top 20 KEGG pathways enriched by differential gut metabolites between ASD and TD children. Count, the number of differential metabolites in the pathway. Ratio=the ratio of number of differential metabolites to all detected metabolites in the

pathway. P value, P value of hypergeometric test. TD = typically developing; ASD = autism spectrum disorders.

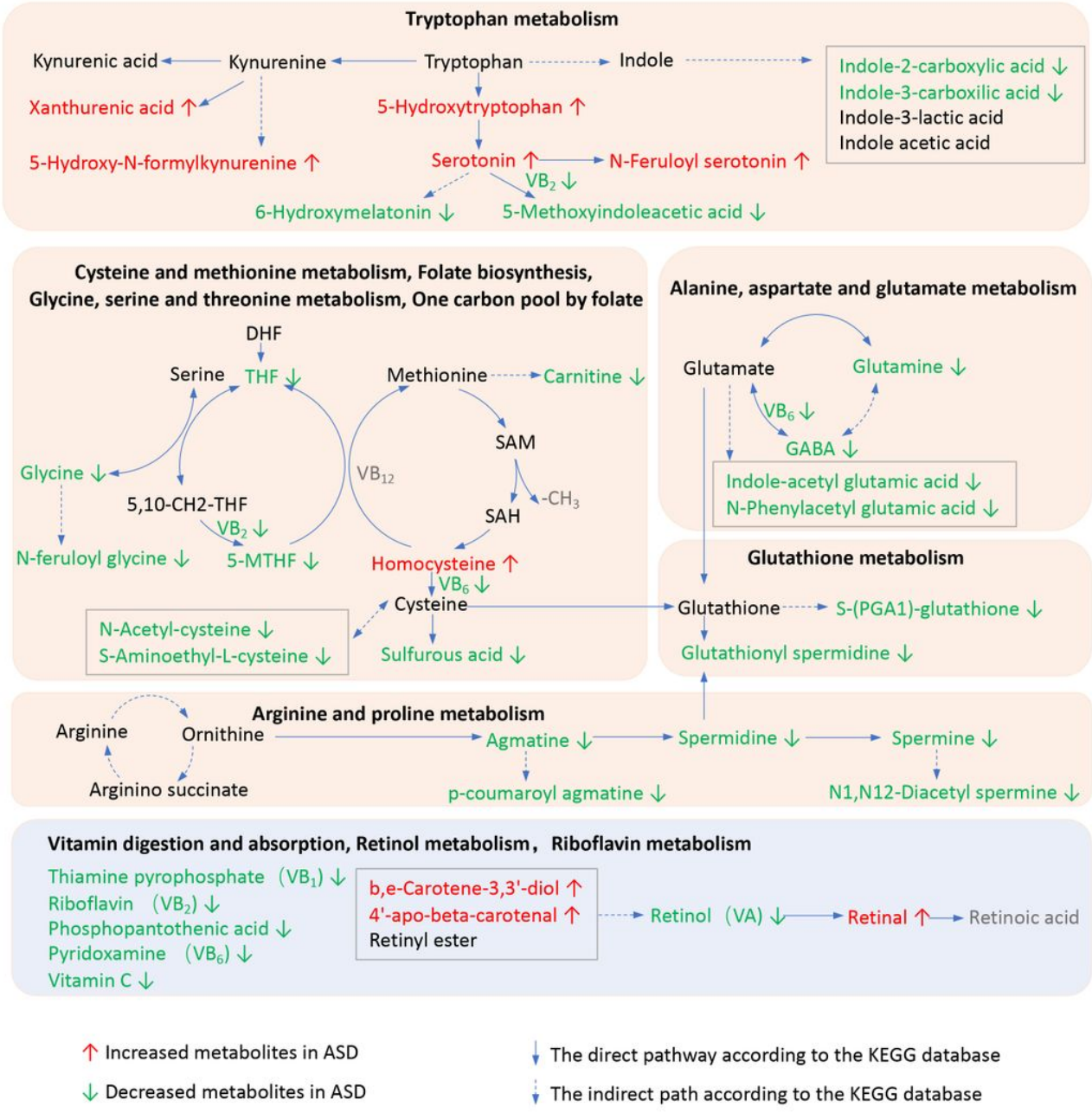

Figure 2

Metabolism pathway networks of the differential metabolites between ASD and TD group Gut metabolites with fold change >1.5, variable important in projection (VIP) >1, and FDR-corrected P values < 0.05 for student's t-test or Mann-Whitney U test were considered significantly differential metabolites between groups. Red font (↑), metabolites increased in ASD group; Green font (↓), metabolites decreased

in ASD group; black font, no significant difference between ASD and TD groups; grey font, undetected. DHF, dihydrofolate; 5-MTHF, 5-methyltrahydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. ASD = autism spectrum disorders.

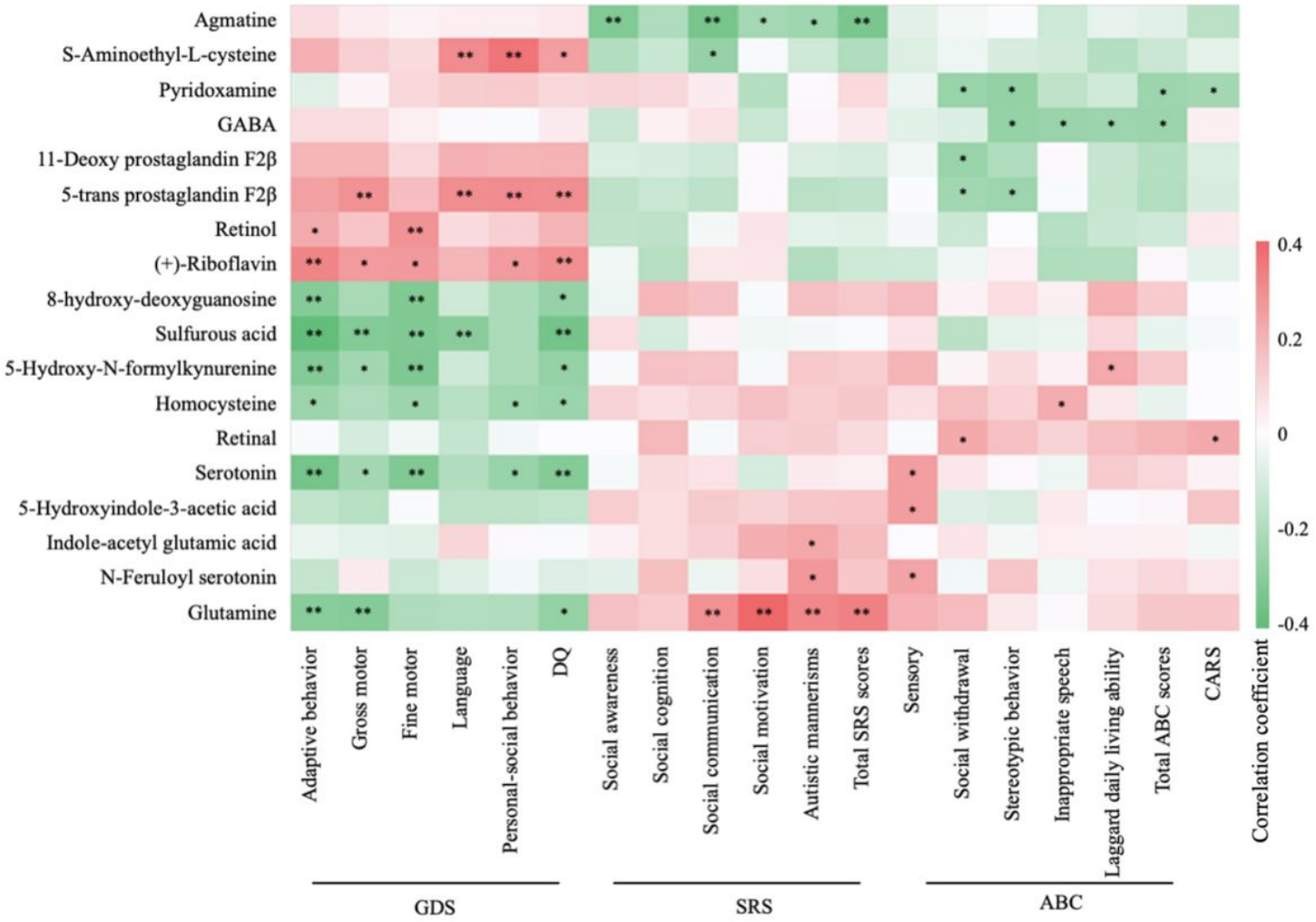

Figure 3

Correlations between gut metabolites with symptoms and neurodevelopment of ASD children The correlation coefficient is indicated by a color gradient from green (negative correlation) to red (positive correlation). The * symbol in each lattice represent a significant correlation. * $P < 0.05$, ** $P < 0.01$. Spearman correlation. GDS=Gesell Developmental Scale, ABC=Autism Behavior Checklist, SRS=Social Responsiveness Scale, CARS=Childhood Autism Rating Scale, DQ=development quotient scores.

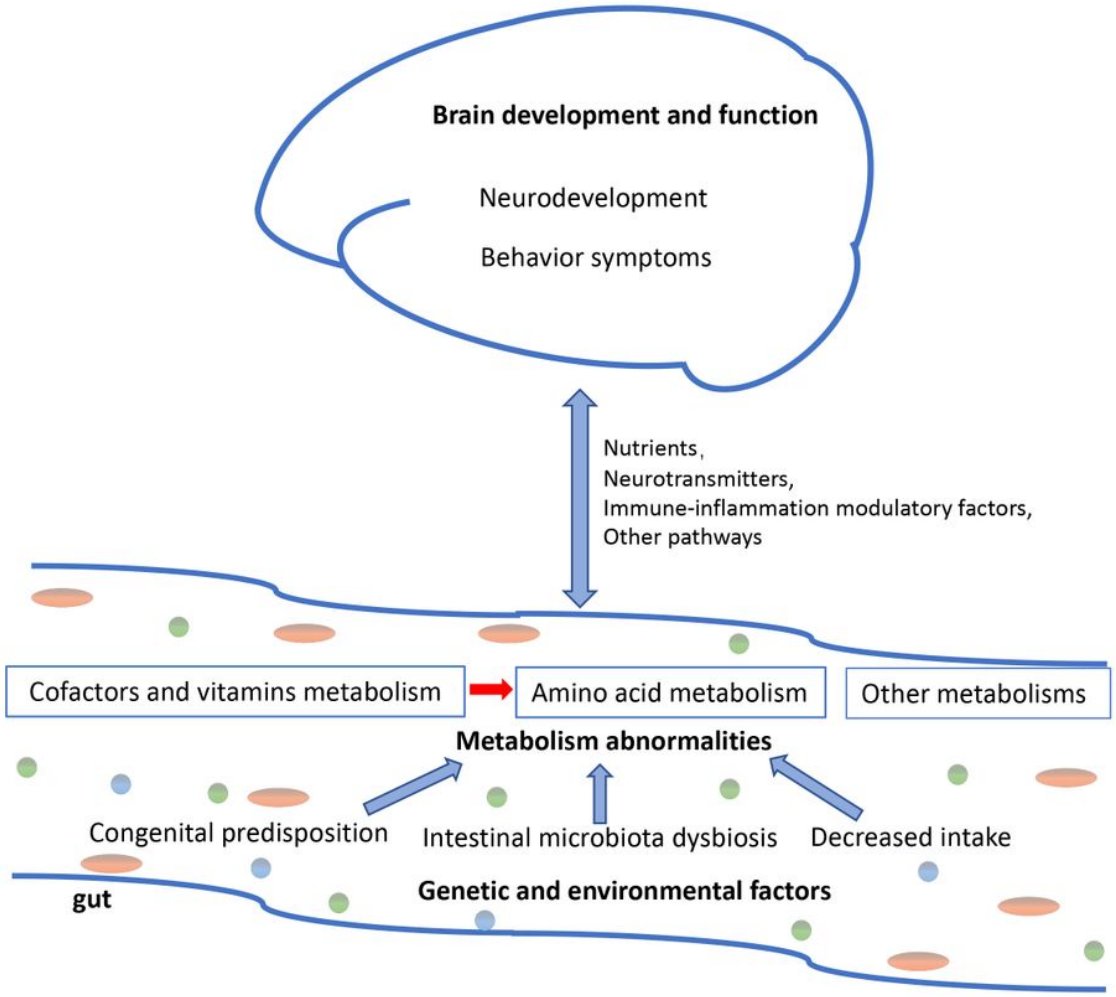

Figure 4

Hypothesis of interplay between the gut metabolism and the gut-brain axis in ASD Aberrant of gut metabolism profiles may be the result of the interaction of multiple factors, including congenital metabolism errors, decreased intake by abnormal eating pattern, and intestinal microflora imbalance. In the interrelated metabolism networks, vitamins metabolism abnormalities and decreased intake of vitamins may disturb the amino acids metabolism, for vitamins B are essential cofactors implicated in multiple biochemical reactions. The metabolites may affect the brain development and function, and subsequently behavior by nutrients, neurotransmitters, immune-inflammation modulatory, and other pathways.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalTable3.xlsx

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- TableS13.docx