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Gut microbial 'TNFα-sphingolipids-steroid hormones' axis in children with autism spectrum disorder: an insight from meta-omics analysis

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Abstract

Background Autism spectrum disorder (ASD) is a persistent neurodevelopmental disorder affecting brains of children. Mounting evidences support the associations between gut microbial dysbiosis and ASD, whereas detailed mechanisms are still obscure.

Methods Here we probed the potential roles of gut microbiome in ASD using fecal metagenomics and metabolomics.

Results Children with ASD were found to be associated with augmented serum cytokines milieu, especially TNFα. Metagenomic analysis generated 29 differential species and 18 dysregulated functional pathways such as *Bifidobacterium bifidum*, *Segatella copri*, and upregulated 'Sphingolipid metabolism' in children with ASD. Metabolomics revealed steroid hormone dysgenesis in children with ASD with lower abundances of metabolites such as estriol, estradiol and deoxycorticosterone. A three-way association analysis showed positive correlations between TNFα and microbial function potentials such as 'Bacterial toxins' and 'Lysosome', indicating the contribution of microbial dysbiosis to neuroinflammation. TNFα also correlated positively with 'Sphingolipid metabolism', which further showed negative correlations with metabolites estriol and deoxycorticosterone. Such results, in consistent with current findings, revealed the contribution of increased TNFα to upregulated sphingolipid metabolism, which further impaired steroid hormone biosynthesis.

Conclusion Our study proposed the gut microbial 'TNFα-sphingolipids-steroid hormones' axis in children with ASD, which may provide new perspectives for developing gut microbiome-based treatments in the future.

Keywords autism spectrum disorder, gut microbiome, metagenomics, metabolomics

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Introduction

Autism spectrum disorder (ASD), also referred to as autism or autistic disorder, is a widespread neurodevelopmental disorder that primarily occurs in early childhood. The global prevalence of ASD is estimated to be around 1% [1], and shows an upward trend in both China and the United States [2, 3]. ASD substantially impacts the physical and mental health of children and adolescents, leading to a high disability rate and the need for long-term rehabilitation. Coupled with the increasing prevalence, ASD imposes considerable economic and psychological burdens on both society and families, and has become a globally recognized public health concern [4]. Nowadays, intervention approaches are still the mainstream treatment options, which require strong expertise and high economic expenditure. Although some medications have been found efficacious for associated symptoms such as aripiprazole and risperidone for irritability, there is still no approved medication for the core symptoms of ASD [5, 6]. Moreover, medications are often associated with side effects [7]. Therefore, new treatments that are safer and more cost-effective are urgently needed.

Currently, strong associations have long been observed between gut microbial dysbiosis and ASD phenotypes, supporting a gut-microbiome-brain connection [8, 9]. Concisely, the host-microbiota crosstalk through the immune system is fundamental to preserve the integrity of the blood brain barrier, which prevents the systematic translocation of bacterial-derived products that can cause microglia hyperactivation within the central nervous system (CNS) and neuroinflammation. Within the central nervous system, bacterial microbes help regulate the development of microglia and other resident immune cells that help sustain and protect neuronal function [10]. Gut microbiota can also produce a variety of neurotransmitters and neuroactive compounds such as short-chain fatty acids that modulate the peripheral and central nervous system, as well as immune system [11, 12]. Such results indicate that gut microbiome is a potential target for treating ASD, which has been confirmed by the success of fecal microbiota transplantation, probiotics and prebiotics in improving immune and phenotypical phenotypes in patients with ASD [13, 14]. However, our understandings on detailed relationships and mechanisms between changes in the microbiome and ASD are remain limited [15], which precludes the develop of precise treatments targeting on gut microbiota for ASD.

To deepen our understandings on the associations between gut microbiome disorder and ASD, we conducted a meta-omics analysis on fecal samples obtained from 26 children with ASD and 26 age-matched neurotypical controls using metagenomics and metabolomics. The findings of this study associating gut microbial dysbiosis with phenotypes of ASD will lay the foundation for developing gut microbiome-targeted treatments in the further for children with ASD.

Materials and methods

Participants and sample collection

Children with ASD (n=26), age- and sex-matched neurotypical children (n=26, controls) were recruited from Lishui Second People's Hospital, Zhejiang, China. The diet, environment, and genetic background of recruited participants were comparable. All children underwent neurological, physical and behavioral examinations. The children with ASD were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5). The diagnosis was confirmed by two experienced developmental and behavioral pediatricians using the Childhood Autism Rating Scale (CARS). Children with ASD that had a history of congenital diseases, acute or chronic affective diseases in the past 3 months were excluded from the study. All the participants who received antibiotics, probiotics, prebiotics or other medications in the past 3 months were also excluded. Feces and blood samples were collected from each participant, and delivered immediately at low temperatures to the laboratory. Feces was transferred and preserved in sterile storage tubes, while blood samples were centrifuged. All samples were stored at -80 °C until analysis. All research was approved by the Ethics Committee of the Lishui Second People's Hospital, Zhejiang, China. Informed written consent was obtained from each of the participants before enrollment.

Measurement of pro-inflammatory factors

Concentrations of proinflammatory factors in serum samples such as IL6, IL1β, and TNFα were quantified with ELISA kit (Invitrogen, USA). Intra-assay and Inter-assay variations for the measurements were all below 10%.

Fecal bacterial DNA extraction and sequencing

Bacterial total DNA from feces was extracted using the QIAamp DNA Micro kit (Qiagen) following manufacturer's instructions. NanoDrop (Thermo Scientific) and agarose gel electrophoresis was utilized to evaluate the concentration and molecular size of bacterial DNA. Then, DNA library was constructed according to manufacturer's instructions (Illumina) including procedures such as cluster generation, template hybridization, isothermal amplification, denaturing and hybridization of sequencing primers. Finally, whole-genome metagenomic sequencing was performed in paired-end mode using Illumina HiSeq 4000 platform at a read length of 350 base pairs (bp).

Quality control and taxonomic profiling

Raw reads were quality controlled using Trimmomatic (v.0.39) to remove adaptors, low-quality reads, bases or PCR duplicates [16]. KneadData (v.0.10.0) was used to remove human DNA contamination through alignment with the human reference genome (GRCh38.p14). High quality reads were then taxonomically profiled at different taxonomic levels using MetaPhlAn4 [17], generating taxonomic relative abundances normalized with total-sum scaling. All the above analyses were conducted with default settings.

Microbial community diversity analysis

The alpha diversity was calculated using R package *vegan*. Wilcoxon rank-sum test was utilized to evaluate the statistical difference of alpha diversity between groups using *stats*. Beta-diversity was estimated based on Bray-Curtis dissimilarity, while the statistical comparison between groups was achieved by the function adonis to perform a permutational multivariate analysis using R package *vegan* with 999 permutations. $P<0.05$ was considered significant.

Function profiling

Microbial gene families and pathway abundances were determined using HUMAnN3 (v.3.6) and the UniRef90 database [18]. Gene families were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database included in HUMAnN3 to obtain KEGG modules and KEGG pathway abundances. Tables of gene family and pathway abundances were normalized to copies per million, including unmapped and unintegrated reads.

Fecal metabolomics analysis

For each participant, 50 mg fecal sample was added to a 2 mL centrifuge tube with 6 mm grinding. After adding 400 uL of the methanol: water (4:1, v/v) solution, the samples were grinded for 6 min at -10 °C and 50 Hz using a Wonbio-96c frozen tissue grinder, followed by a low-temperature ultrasonic extraction at 5 °C and 40 kHz for 30 min. The samples were then stored at -20 °C for 30 min, and centrifuged at 4 °C and 13,000 g for 15 min. Equal volumes of supernatant from each sample were pooled to obtaine quality control (QC) samples for system conditioning and quality control. The remaining clear supernatant was collected and transferred to an injection vial for liquid chromatography-mass spectrum/mass spectrum (LC-MS/MS) analysis.

The LC-MS/MS analysis was conducted using a Waters (Milford, MA, United States) ACQUITY UPLC system, equipped with an ACQUITY UPLC BEH C18 column. Mass spectrometric data were acquired using a Waters Q-TOF Premier mass spectrometer in both positive and negative modes. QC samples were injected every six samples through the analytical process. Raw LC/MS data were processed with MarkerLynx Applications Manager (version 4.1, Waters, Milford, MA, United States), which detected, integrated, normalized the intensities of the peaks within the sample, and generated a three-dimensional data matrix including sample information, metabolite names, and mass spectral response intensity. Metabolites were identified using human metabolome database (HMDB) and Metlin. For further analysis, metabolic features with a relative standard deviation greater than 30% in QC samples were excluded.

Integrating metabolome, microbiome and phenotypes

A three-way correlation analysis was utilized to screen potential KEGG modules that correlated significantly with differential metabolites and important phenotypes. In the three-way analysis, correlations between the functional potential, metabolites and phenotypes were estimated by Spearman's correlation.

Statistical analysis

Numerical variables were expressed as median and inter-quantile range (IQR). Categorical variables were expressed as ratio (number of male/26). Wilcoxon rank-sum test and chi-square test were used for comparison between two groups for numerical and categorical variables, respectively. Serum cytokine profiles between groups was demonstrated using the score plot of principal component analysis. Similarities of serum cytokines and metabolomic profiles between groups were statistically assessed by the function anosim using R package *vegan* with Bray-Curtis distance and 999 permutations. Differences in each cytokine between groups was estimated using Wilcoxon rank-sum test. For metabolomics and metagenomics data, features detected in more than 80% of samples were retained for further analysis. Then, the Linear discriminant analysis Effect Size approach (LEfSe, $P<0.05$, LDA > 2) was utilized to obtain differentially abundant metabolites, microbiota and functional potentials [19]. The pathway enrichment analysis of ASD-associated metabolites was conducted using the statistical analysis system module of MetaboAnalyst 5.0 [20]. To determine if metabolome and serum phenotypes were associated, a mantel test was performed using the *mantel* function from the R package *vegan*. Bray-Curtis dissimilarity matrix based on differential species was computed to perform this test. To evaluate whether metabolome and microbiome were associated, a *mantel* test was also conducted using Bray-Curtis dissimilarity matrices based on differential species and abundance table of differential metabolites.

Results

Clinical characteristics of children with ASD

To probe the potential roles of gut microbiome in children with ASD, we recruited 26 ASD, and 26 age- and sex-matched neurotypical children. Serum and fecal samples were collected from each participant. The ages of ASD and neurotypical children were 5.0 (4.0, 5.0) and 5.0 (5.0. 5.8). The numbers of male participants in ASD and non-autistic group were 20/26 and 13/26, respectively. No significant difference in age or sex was observed between the two groups ($P>0.05$). Furthermore, we found that the serum cytokine profile of ASD children differed significantly from that of neurotypical children (Fig. 1a, $P=0.02$). Among them, only TNFα showed a significant higher level in children with ASD ($P<0.01$, Fig. 1b). This is consistent with previous findings that children with ASD displayed an augmented inflammatory cytokine milieu [21].

Gut microbial community disorder in children with ASD

To investigate the associations between gut microbiome and ASD, we performed whole-genome metagenomic sequencing on fecal samples collected from children with ASD and neurotypical children, generating around 416 Gbp of sequencing data on average (8 Gbp per sample). No significant difference was observed in Shannon index ($P=0.05$) or Simpson index ($P=0.17$) between two groups, although a slight decrease in both indexes was observed in children with ASD (Fig. 1c and d). The difference in gut microbial community in terms of β-diversity was not so significant ($P=0.06$), either.

To uncover whether any bacterial species was potentially associated with ASD, we adopted LEfSe algorithm that considers both *P* values from Kruskal-Wallis test and LDA scores from linear discriminant analysis. This analysis led to the identification of 9 genera including Ruminococcus, Mediterraneibacter, Fusicatenibacter, Roseburia, Candidatus_Cibionibacter, Eubacteriales_unclassified, GGB9699, Brotolimicola, and Schaalia that were down-regulated in children with ASD, with the relative abundances of them shown in Fig. 1e. Moreover, the abundances of 13 species such as *Fusicatenibacter saccharivorans*, *Mediterraneibacter faecis*, *Roseburia faecis*, *Blautia faecis* and *Candidatus Cibionibacter quicibialis* were significantly lower in children with ASD (Fig. 1f). In terms of KEGG function potentials, six pathways including 'Bacterial toxins', 'Sphingolipid metabolism', 'Lysosome' were more abundant, while pathways such as 'Prokaryotic defense system', 'Two component system', 'Bacterial motility proteins' were less abundant in

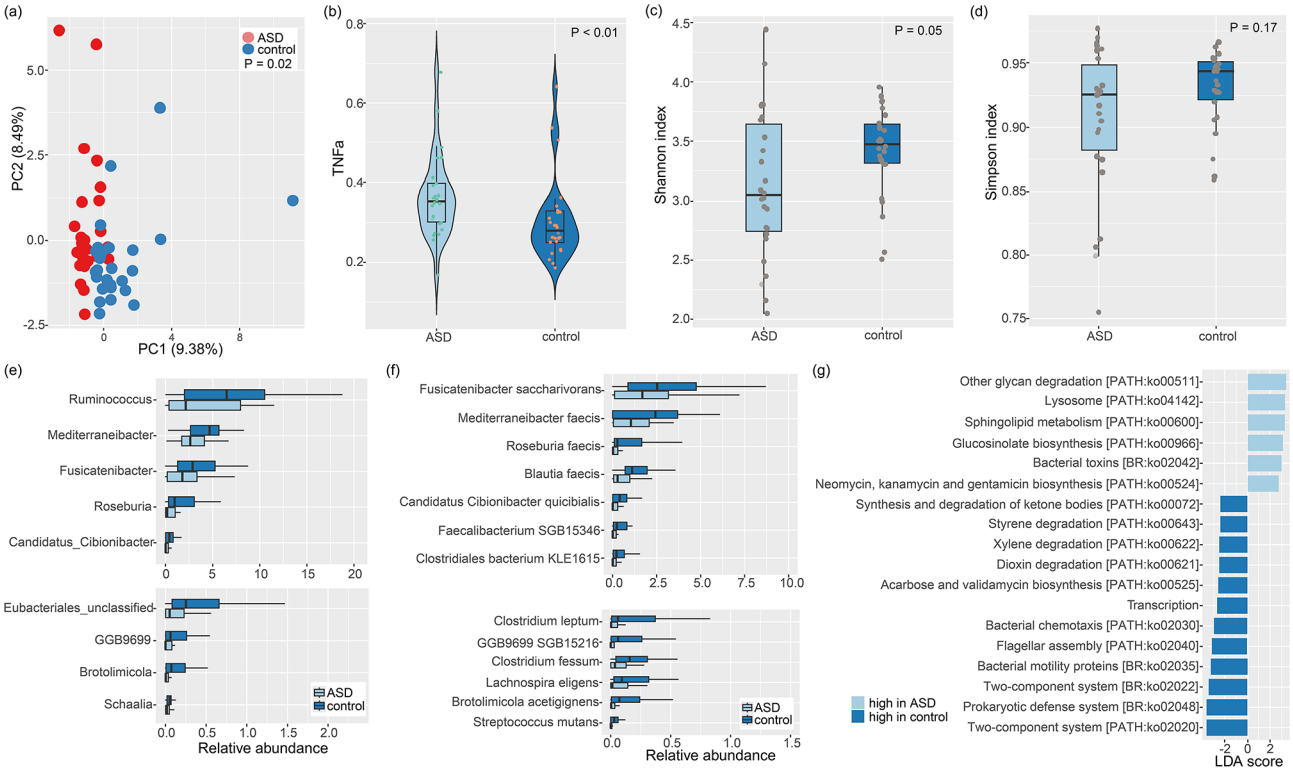

Fig. 1 (**a**) Score plot of serum cytokines based on principal component analysis. (**b**) Serum levels of TNFα in children with ASD and controls. (**c**) Shannon index of gut microbial species in children with ASD and controls. (**d**) Simpson index of gut microbial species in children with ASD and controls. (**e**) Boxplot illustrating the relative abundance of genera differential between groups. (**f**) Boxplot showing the relative abundance of species differential between groups. (**g**) Bar plot showing LDA scores of microbial function potentials differential between groups

children with ASD (Fig. 1g). Such results indicated that gut microbial community was disordered in terms of both taxonomic and functional compositions in children with ASD.

Fecal metabolic dysregulation in children with ASD

We next performed untargeted metabolomics on fecal samples of all participants to investigate whether fecal metabolome was dysregulated in children with ASD. In total, we measured more than 1000 metabolites. Examinations of the metabolites showed a small but significant change between groups ($P=0.02$), where significant higher dissimilarity ranks in children with ASD compared to controls were observed (Fig. 2a). At the level of individual metabolites, 198 metabolites were significantly perturbed in children with ASD compared to neurotypical controls ($P<0.05$, LDA score>2). The score plot of principal component analysis demonstrated a significant difference in the overall abundances of the 198 metabolites between groups ($P<0.01$, Fig. 2b). Among them, 159 metabolites were down-regulated, while only 39 metabolites were up-regulated in children with ASD. The relative abundances of the 8 most abundant differential metabolites were shown in Fig. 2c, while the abundances of the others were provided in Figures S1. We then enriched them to corresponding metabolic pathways based on KEGG database, respectively. It was interesting to find that the down-regulated metabolites were enriched to pathway 'Steroid hormone biosynthesis', while the up-regulated ones were enriched to 'Linoleic acid metabolism' and 'Valine, leucine and isoleucine biosynthesis' (Fig. 2d and e, $P<0.05$, FDR<0.05). A close inspection revealed the drastic decrease in the abundance of eight metabolites enriched in pathway 'Steroid hormone biosynthesis' in all children with ASD and controls (Fig. 2f). The abundance of the above metabolites in male and female participants was shown in Figure S2a and S2b, respectively. The abundance of the four metabolites enriched in pathway 'Linoleic acid metabolism' in both groups of children was shown in Figure S2c. In a word, fecal metabolome was also dysregulated in children with ASD.

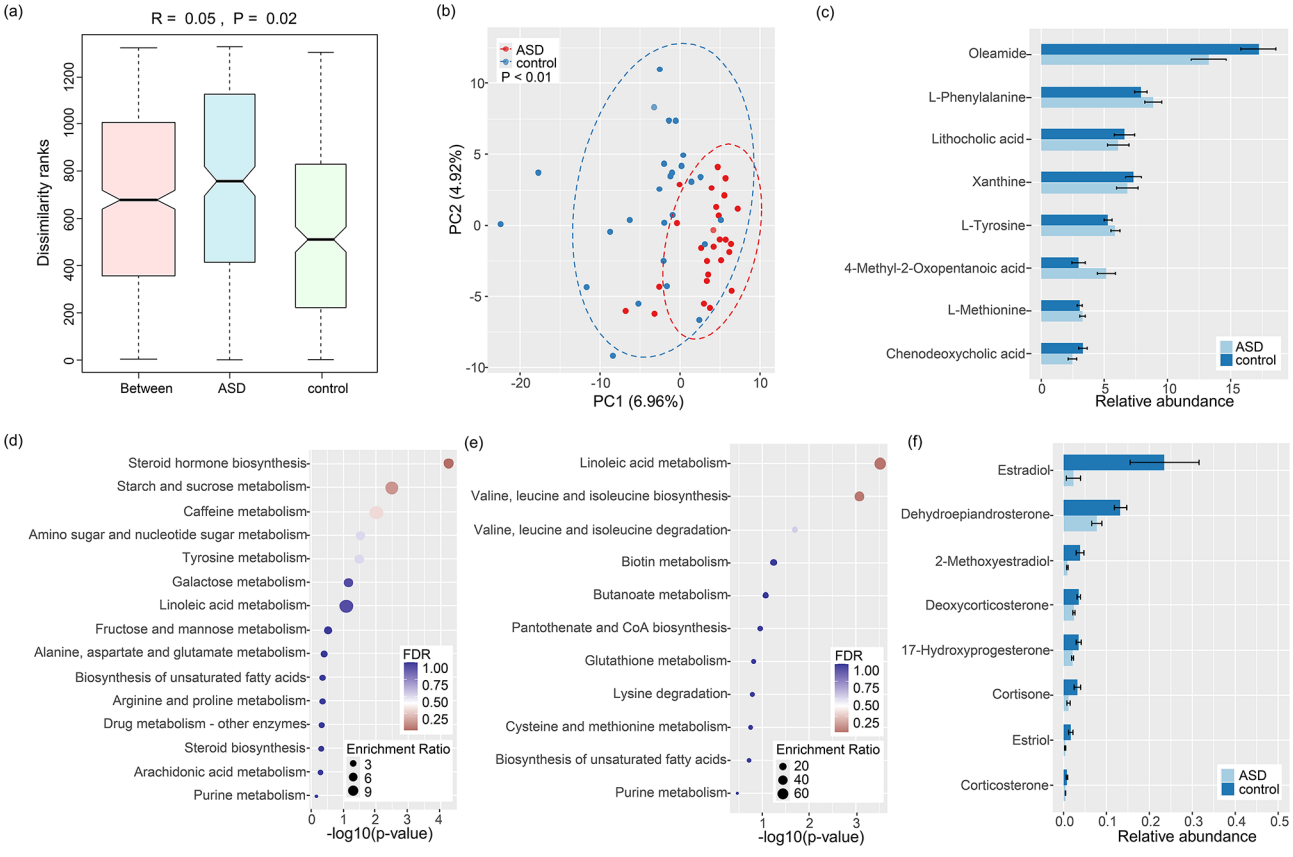

Fig. 2 (**a**) The dissimilarity ranks between and within classes based on ANOSIM analysis of overall metabolites. (**b**) The score plot of principal component analysis based on 198 metabolites differential between groups. (**c**) The relative abundances of 8 most abundant differential metabolites in children with ASD and neurotypical controls. (**d**) KEGG pathways enriched by metabolites with lower abundances in children with ASD. (**e**) KEGG pathways enriched by metabolites with higher abundances in children with ASD. (**f**) Relative abundances of metabolites enriched in KEGG pathway 'Steroid hormone biosynthesis' in all participants

Three-way associations between gut microbiome, metabolites and serum cytokines

The above results showed dysregulated gut microbial community, fecal metabolome and host serum cytokines in children with ASD. To entangle the internal relationships among them, mantel tests were firstly conducted. Based on mantel test, we observed significant correlations ($P<0.01$) between differential bacteria and the overall serum cytokine profiles. We further correlated the abundance of all significant bacterial species with a panel of serum cytokines, and found significant negative correlations between *Faecalibacterium SGB15346*, *Streptococcus mutans* and TNFα (Fig. 3a). Such results indicated that gut microbial dysbiosis was associated with the augmented inflammatory cytokine milieu in children with ASD. Further analyses based on mantel tests revealed significant correlations between differential species and dysregulated microbial functions (Fig. 3b). Association was also observed between differential species and differential metabolites (Fig. 3b). This led to the assumption that gut microbial dysbiosis may contribute to ASD via dysregulated function potentials.

To investigate the internal relationships among gut microbiome, metabolites and serum cytokines in children with ASD, a three-way association analysis was conducted. It was shown that microbial function potentials upregulated in children with ASD including 'Bacterial toxins', 'Lysosome', 'Sphingolipid metabolism', 'Other glycan degradation', and 'Neomycin, kanamycin and gentamicin biosynthesis' showed positive correlations with TNFα (Fig. 3c). The relative abundance of the above function potentials was shown in Fig. 3d. This implied the contribution of gut microbial dysbiosis to augmented inflammatory cytokine milieu, especially increased TNFα, via dysregulated function potentials. Among the above microbial function potentials, 'Sphingolipid

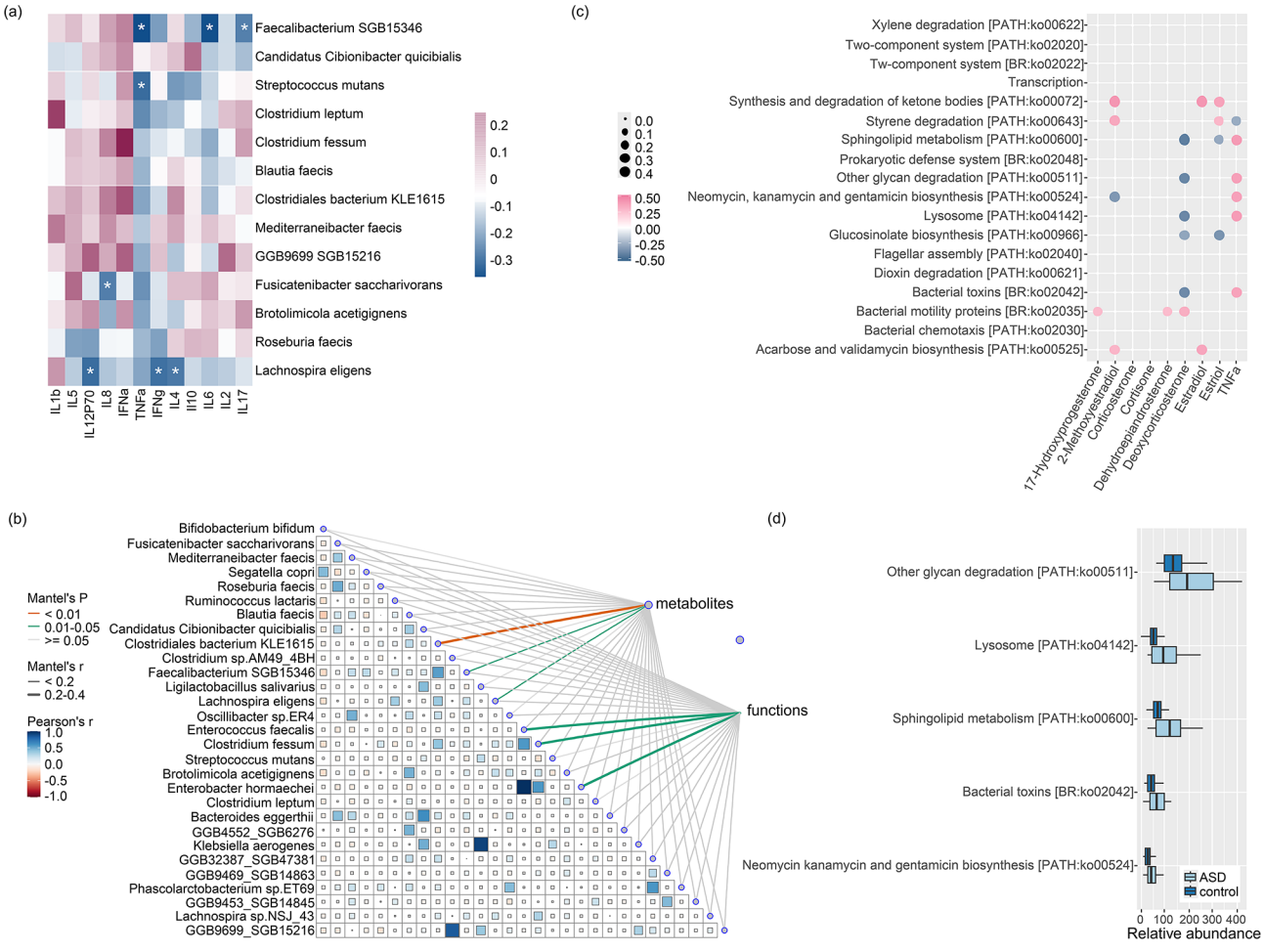

Fig. 3 (**a**) Heatmap illustrating the correlations between differential species and serum cytokines. (**b**) Associations between differential species and microbial functions, differential metabolites. The lower triangle shows the associations between differential species, while the lines demonstrate the correlations between differential species and differential metabolites, as well as the correlations between differential species and microbial functions. (**c**) Scatter diagram showing the associations between differential microbial function potentials, metabolites enriched in pathway 'Steroid hormone biosynthesis', and TNFα. (**d**) The relative abundance of microbial function potentials correlated positively with TNFα

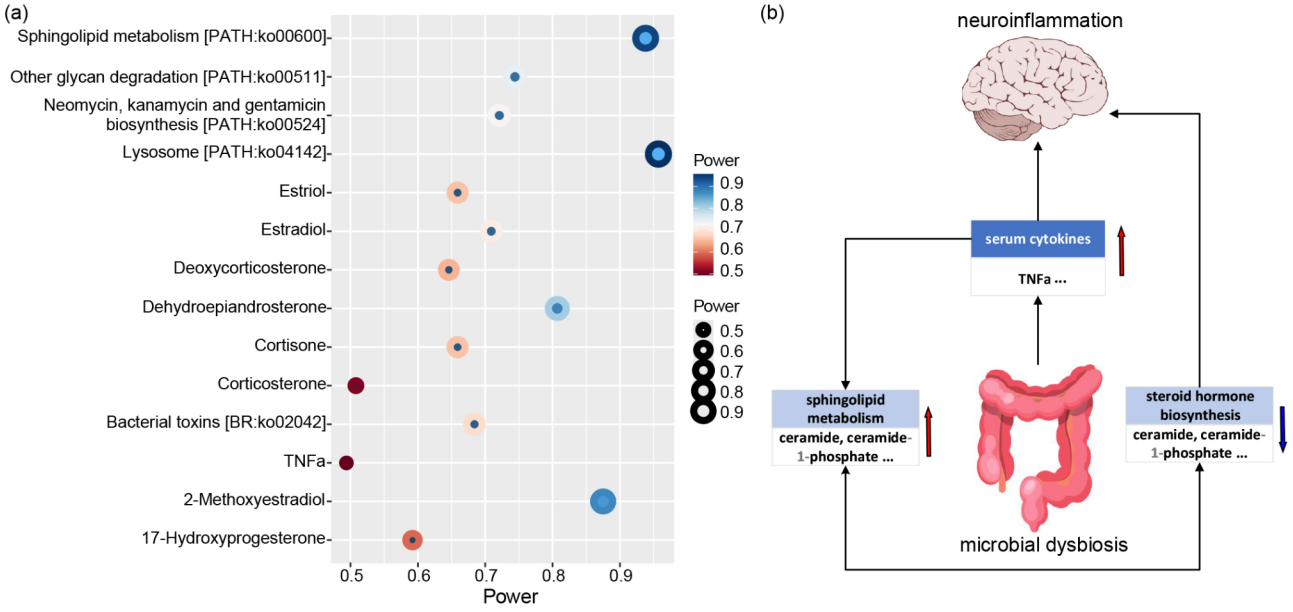

Fig. 4 (**a**) The statistical power of differential cytokines, differential microbial function potentials, and differential metabolites involved in the gut microbial 'TNFα-sphingolipids-steroid hormones' axis. (**b**) A schema showing the gut microbial 'TNFα-sphingolipids-steroid hormones' axis

metabolism', 'Other glycan degradation', 'Lysosome' and 'Bacterial toxins' correlated negatively with deoxycorticosterone, while 'Sphingolipid metabolism' and 'Glucosinolate biosynthesis' correlated negatively with estriol. Moreover, microbial function potential 'Bacterial motility proteins', that was downregulated in children with ASD, correlatively positively with deoxycorticosterone, dehydroepiandrosterone, and 17-hydroxyprogesterone. Such results indicated that dysregulated microbial function potentials may also contribute to steroid hormone dysgenesis observed from microbial metabolites.

We further probed how microbial function dysregulation was associated with ASD and steroid hormone dysgenesis. Among the microbial function potentials, 'Sphingolipid metabolism', upregulated in children with ASD (Fig. 3d), was found to correlate positively with TNFα (Fig. 3c). Meanwhile, 'Sphingolipid metabolism' also showed negative correlations with estriol, and deoxycorticosterone (Fig. 3c). Such results led to the assumption that dysregulated 'Sphingolipid metabolism' might be a mediator that bridged microbial dysbiosis and impaired 'Steroid hormone biosynthesis' in children with ASD. As a confirmation of the steroid hormone dysgenesis observed in microbial metabolites, we compared the steroid hormone-related microbial function potentials between groups. We found that the function potential 'Estrogen signaling pathway' was lower in children with ASD compared to controls ($P<0.05$, Figure S3a). Although not significant, the apparent downward trend in children with ASD compared to controls to some extent indicated the impaired microbial function potential 'Steroid hormone biosynthesis' in children with ASD (Figure S3b). We also evaluated the level of sphingolipid-related metabolites between groups. Other than the significant decrease of NBD-dihydro-ceramide in children with ASD (Figure S3c), the upward trend of microbial metabolite ceramide-1-phosphate was in line with the upregulated microbial function potential 'Sphingolipid metabolism' in children with ASD (Figure S3d). Such results supported the presence of dysregulated steroid hormone-related microbial function potentials and sphingolipid metabolism disorder in children with ASD, and implied the central role of sphingolipid metabolism in linking ASD and steroid hormone dysgenesis.

Considering the limited sample size of this study, we further calculated the statistical power of differential cytokines, differential microbial function potentials, and differential metabolites involved in the gut microbial 'TNFα-sphingolipids-steroid hormones' axis. As shown in Fig. 4a, nearly all comparisons were conducted with a power larger than 0.5. For central members of the axis, such as microbial function potential 'Sphingolipid metabolism', steroid hormone-related metabolites 2-methoxyestradiol and dehydroepiandrosterone, the powers were larger than 0.8. Such results indicated that the statistical power for the comparison results that supported the findings of the gut microbial 'TNFα-sphingolipids-steroid hormones' axis in this study was acceptable. Therefore, the meta-omics analysis in our study proposed the potential role of gut microbial 'TNFα-sphingolipids-steroid hormones' axis in ASD (Fig. 4b).

Discussion

Based on a meta-omics study using metagenomics and fecal metabolomics, we revealed augmented inflammatory cytokine milieu, dysregulated microbial functional potentials, and microbial metabolic disorders in children with ASD. A three-way association analysis showed the associations among TNFα, 'Sphingolipid metabolism' and steroid hormone dysgenesis, implying the presence of gut microbial 'TNFα - sphingolipids- steroid hormones' axis in children with ASD.

TNFα has been identified as a key molecule in neuroinflammation associated with ASD. Studies suggest that dysregulated neuroinflammation, particularly involving TNFα, may be implicated in the pathophysiology of ASD [22, 23]. The significant increase of TNFα observed in this study confirmed the presence of neuroinflammation in children with ASD. The significant association between differential species and serum cytokines confirmed the potential role of gut microbial dysbiosis in neuroinflammation of patients with ASD [24]. Nowadays, multiple assumptions have been proposed to explain how gut microbial dysbiosis contributes to neuroinflammation in ASD via 'gut-brain' axis, such as fecal metabolites that can affect the activation of immune cells, and intestinal barrier dysfunction that allows entrance of bacteria and microbial products into host [25]. Our study observed significant positive correlations between microbial functional potentials (such as 'Bacterial toxins' and 'Lysosome') and TNFα. Bacterial toxins have been reported to manipulate the host's immune system, leading to the production of inflammatory cytokines such as TNFα [26]. Several studies have demonstrated a positive correlation between the lysosome pathway and TNFα, highlighting the role of lysosomes in regulating the inflammatory processes associated with TNFα [27, 28]. Although the relationship between abnormalities in the sphingolipid metabolism and ASD has not been thoroughly explored, abnormal sphingolipid metabolism has been implicated in many inflammatory and immune processes [29]. Moreover, it has been suggested that important sphingolipid metabolites may be promising biomarkers for the diagnosis of ASD [29]. Thus, it is reasonable to propose that the dysregulated bacterial functions resulting from microbial dysbiosis may contribute to the increased level of TNFα and neuroinflammation in children with ASD.

Other than disordered gut microbial community and serum cytokines, metabolomic analysis of the study revealed dysregulated microbial metabolic capacities in children with ASD. Specifically, the levels of metabolites enriched in pathway 'Steroid hormone biosynthesis', including estradiol, 2-methoxyestradiol, estriol, dehydroepiandrosterone, 17-hydroxyprogesterone, cortisone, corticosterone, and deoxycorticosterone, were significantly lower in fecal samples of children with ASD. Estradiol, the bioactive form of estrogen, has a neuroprotective effect [30]. Lower levels of estradiol were found to be associated with high odds of autism [31]. Although no studies directly mentioned the associations between glucocorticoids such as deoxycorticosterone and ASD, several studies reported that higher cortisol (a glucocorticoid) levels were associated with lower odds of ASD [31]. In other words, metabolomic analysis of this study reported the presence of steroid dysgenesis in children with ASD, which has been reported to be associated with male patients with ASD [32]. Moreover, estrogen signaling has been proposed as a therapeutic target in neurodevelopmental disorders [33]. Corticosteroid treatment was found to be associated with an increased frequency of modulated evoked response, reduction in response distortion, and improvements in language and behavior scores [34]. Such results in together pointed out the value of correcting steroid hormone dysgenesis in treating ASD.

To uncover the driving forces underlying steroid hormone dysgenesis, a mantel test between differential bacteria and metabolites enriched in 'Steroid hormone biosynthesis' was conducted. A P value less than 0.01 confirmed the contribution of microbial dysbiosis to steroid hormone dysgenesis. A three-way association analysis revealed that microbial functional potential 'Sphingolipid metabolism' showed negative correlations with metabolites estriol and deoxycorticosterone, and a positive correlation with TNFα. Steroid hormones are synthesized mainly via cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling pathway including members such as steroidogenic factor 1 (SF1) and cytochrome P450 family 17 (CYP17). Recent studies have identified important roles of bioactive sphingolipids such as ceramide and sphingosine in the homeostasis of steroid hormones [35]. Ceramide is reported to negatively regulate progesterone production, and to suppress human choriogonadotropin-stimulated testosterone production [36, 37]. Ceramide can also regulate the expression and activity of steroid synthase, such as regulating P450c17α and inhibiting cAMP production [37]. Sphingosine is confirmed as an antagonist of SF-1, which in combination with SF-1 leads to a decrease of CYP17 expression [38]. Therefore, the up-regulated microbial function potential 'Sphingosine metabolism' may contribute to dysgenesis of microbial steroid hormones in children with ASD.

Recent studies found not only sphingolipids can modulate steroid hormone secretion, but also steroid hormones can control sphingolipid metabolites [35]. TNFα can activate sphingomyelinases and generate intracellular ceramide [39], a mediator in cytokine and growth factors signaling pathways, ultimately leading to a change in basal steroid hormone production [40]. It has been

reported that TNFα signaling can regulate steroid hormone production through sphingolipid metabolism [41]. Moreover, sphingolipids can act as signaling molecules in immune response involving TNFα [42]. These studies collectively indicate that sphingolipids, TNFα and steroid hormones are intricately linked in the pathophysiology of various diseases including ASD. Combining all the above findings, our study proposed that gut microbial 'TNFα-sphingolipids-steroid hormones' axis can act as a potential target in treating ASD. However, the sample size is quite limited in this study, further studies should be conducted to confirm the findings of the study. Further studies should also seek a deeper understanding of the bacteria that are implicated in the axis, which will lay the foundation for developing microbiota-targeted treatments for ASD.

Conclusions

Based on a meta-omics analysis, we revealed the presence of augmented inflammatory cytokine milieu, dysregulated microbial community, and microbial metabolic disorders in children with ASD. Further three-way association analysis revealed intricate links among TNFα, sphingolipids and steroid hormones in the pathophysiology of ASD in children, and proposed the potential axis of gut microbial 'TNFα-sphingolipids-steroid hormones' in ASD. Considering the relative limited sample size of this study, further studies with larger sample sizes are still required. Future efforts should also be made to uncover the specific bacteria that can act as targets in treating ASD from the gut microbial 'TNFα-sphingolipids-steroid hormones' axis.

Abbreviations

TNFα	Tumor necrosis factor alpha
ASD	Autism spectrum disorder
CNS	Central nervous system
KEGG	Kyoto Encyclopedia of Genes and Genomes Orthology database
QC	Quality control
LC-MS/MS	Liquid chromatography-mass spectrum/mass spectrum
HMDB	Human metabolome database
IQR	Inter-quantile range
LEfSe	Linear discriminant analysis Effect Size
SF1	Steroidogenic factor 1
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
CYP17	Cytochrome P450 family 17

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-024-05973-3.

Supplementary Material 1: **Figure S1.** The relative abundances of the remaining differential metabolites in children with ASD and neurotypical controls.

Supplementary Material 2: **Figure S2**. (a) Relative abundances of metabolites enriched in KEGG pathway 'Steroid hormone biosynthesis' in male participants. (b) Relative abundances of metabolites enriched in KEGG pathway 'Steroid hormone biosynthesis' in female participants. (c) Relative abundances of metabolites enriched in KEGG pathway 'Linoleic acid metabolism' in all participants.

Supplementary Material 3: **Figure S3**. (a) Boxplot showing the relative abundance of microbial function potential 'Estrogen signaling pathway'. (b) Boxplot showing the relative abundance of microbial function potential 'Steroid hormone biosynthesis'. (c) Bar plot showing the intensity of metabolite NBD-dihydro-ceramide. (d) Bar plot showing the intensity of metabolite ceramide-1-phosphate.

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Author contributions

Conceptualization: ShiweiYe, Zongxin Ling, Li Shao; Sample collection: Guangyong Cai, Jinlong Fu, Weishi Zhang, Yuefang Ye; Data analysis: Li Shao; Manuscript writing: Li Shao; Writing-review and editing: Li Shao, ShiweiYe, Zongxin Ling.

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Data availability

The dataset supporting the conclusions of this article is available in the Sequence Read Archive repository (PRJNA1141738).

Declarations

Ethics approval and consent to participate

All research was approved by the Ethics Committee of the Lishui Second People's Hospital, Zhejiang, Chia. Informed written consent was obtained from each of the participants before enrollment.

Consent for publication

All the authors approve the publication of this article and this version of the manuscript.

Competing interests

The authors declare no conflicts of interest.

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