

A Combined Proteomics and Metabolomics Profiling to Investigate the Genetic Heterogeneity of Autistic Children

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Research

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Abstract

Background: Autism spectrum disorder (ASD) has become one of the most common neurological developmental disorders in children. However, the study of ASD diagnostic markers faces significant challenges due to the existence of heterogeneity.

Methods: In this study, genetic testing was performed on children who were clinically diagnosed with ASD. Children with ASD susceptibility genes and healthy controls were studied. The proteomics of plasma and peripheral blood mononuclear cells (PBMCs) as well as plasma metabolomics were carried out.

Results: The results showed that although there was genetic heterogeneity in children with ASD, the differentially expressed proteins (DEPs) in plasma, PBMCs, and differential metabolites in plasma could still effectively distinguish autistic children from controls. The mechanism associated with them focus on several common and previously reported mechanisms of ASD.

Limitations: The number of samples carrying risk genes in omics research is limited. In further research, a large sample size is required. A group of children who have been diagnosed with ASD but have not been detected to carry risk genes should also be included.

Conclusion: The biomarkers for ASD diagnosis could be found by taking DEPs and differential metabolites into consideration. Integrating omics data, glycerophospholipid metabolism and N-glycan biosynthesis might play a critical role in the pathogenesis of ASD.

Introduction

Autism spectrum disorder (ASD) is a group of developmental neurological disorders characterized by early onset of abnormal social communication and restricted repetitive behaviors and interests. In recent years, its incidence has gradually increased. ASD is about four times more common among boys than girls¹. Although its pathogenesis has not been clarified, studies have shown that it may be a multifactorial disorder. Twin studies suggest genes play a key role in the pathogenesis of ASD². It is estimated that ASD may involve thousands of genes¹. However, ASD is highly heterogeneous, with each gene individually accounting for less than 1% of cases³. ASD may also be caused by the environmental factors. Therefore, it may be caused by interaction between genes and environmental factors¹.

Currently, there is no specific treatment for ASD. Studies show that early intervention can significantly improve ASD symptoms of diagnosed children and help them transition into society^{4,5}. Early diagnosis is crucial for early detection and early intervention for autistic children. However, at present, there is a lack of effective biomarkers for clinical diagnosis^{1,6-8}. The diagnosis of ASD is based on the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V)⁹. This standard brings a certain level of subjectivity into diagnosis, and is not entirely conducive to early detection of the disease.

The plasma contains abundant substances that are convenient for sampling. Thus, it becomes a suitable candidate when screening diseases. In the search for reliable biomarkers for ASD, some studies on blood protein- or metabolites-based biomarkers have been carried out^{1,6-8}. Some other studies suggest that peripheral blood cells such as peripheral blood mononuclear cells (PBMCs)^{10,11} and lymphoblastoid cell lines (LCLs)¹² may become useful tools to investigate systemic neurochemical changes in ASD. However, due to the existence of heterogeneity, finding peripheral blood or cell markers for the diagnosis of ASD can be challenging. Heterogeneity in etiology, phenotype, and outcome are hallmarks of ASD¹³. This may be due to the fact that ASD is a multifactorial disease. Heterogeneity complicates the quest for personalized medicine in ASD. Combining data from autistic children with different etiologies may lead to confusing results. Factors such as genetic variation, comorbidities, and gender contribute to the heterogeneity of ASD. Among them, genetic variation is the main contributor.

To explore the influence of gene heterogeneity on the search for diagnostic markers of ASD, we performed genetic testing on 122 children clinically diagnosed with ASD in the present study. Five children of the sample were found to be carrying ASD susceptibility genes. The plasma and PBMCs of these children and their gender- and age-matched healthy controls were collected, plasma and PBMCs proteomics and plasma metabolomics analysis were carried out. Based on protein and metabolite profiles collected, the diagnostic markers and the pathogenesis of ASD were studied (Fig. 1).

Materials And Methods

A new generation of sequencing technology for targeted sequencing of autistic candidate genes

An overview of the workflow used in this study is shown in Fig. 1. One hundred and twenty-two autistic children (2–6 years old) were recruited from the Maternal and Child Health Hospital of Baoan in Shenzhen, China. The diagnosis of ASD was based on the criteria of autistic disorders as defined in the DSM- \AA by a child neuropsychiatrist.

EDTA (Ethylenediaminetetraacetic acid) anticoagulation tubes were used to collect 4 mL of peripheral fasting blood from children in the morning. Genomic DNA was extracted from fresh blood samples using the phenol-chloroform extraction method. DNA samples were stored in anhydrous ethanol dissolved in TE buffer and diluted to 50ng / μ L for subsequent experiments. The molecular inversion probe (MIP) was used to design probes for autism risk genes as previously described¹⁴. The target genes were captured, digested, and amplified by PCR (Polymerase chain reaction). After enrichment and purification of the product, high-throughput sequencing was carried out on a hiseq 3000 platform (Illumina, USA). The original sequencing data were then processed by quality control, comparison, and filtration. The mutation sites were annotated and analyzed for function.

Proteomics analysis of plasma and peripheral blood mononuclear cells

Protein sample preparation

Subsequently, we performed plasma and PBMCs proteomics studies on the children with *de novo* mutations, along with gender- and age-matched healthy controls (**Table S1**). 4mL of fasting blood samples were collected in EDTA-coated plastic tubes. Plasma was separated by centrifugation at 800× g for 10 min at 4 °C, and the supernatant was collected and centrifuged at 10,000×g for 30 min at 4 °C, and then divided into aliquots and stored immediately afterwards at -80°C. PBMCs were separated by density gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA)¹¹.

For proteomics analysis, the plasma samples were processed using Multiple Affinity Removal Column (Hu6; MARC; 4.6×50 mm, Agilent, Palo Alto, CA, USA) as previously described¹⁵. PBMCs were lysed in RIPA lysis buffer (Beyotime, Shanghai, China), sonicated 10 times for 5s with 10s pause interval in an ice-water bath, and then centrifuged at 10,000×g at 4°C for 60 min¹¹. The supernatant was taken away and stored at -80°C until use. The protein concentrations were determined and optimized by Bradford assay.

iTRAQ labeling and high-pH RPLC fractionation

The iTRAQ analysis was performed according to the previous study^{11,15}. The protein extraction (100µg) of plasma or PBMCs from each child with ASD and controls was reduced, alkylated, and digested with trypsin (Promega, Madison, WI, USA) at a ratio of 1:30 at 37°C overnight, and then labeled with iTRAQ reagents (AB Sciex, Foster City, CA, USA). The information of iTRAQ tags for plasma and cells is shown in **Table S2**. iTRAQ analysis included set A and set B. After labeling, these were incubated at room temperature for 2h, and then mixed and lyophilized. The dried samples were reconstituted in 100µL double-distilled water (ddH₂O) and injected into the Agilent HPLC (high-performance liquid chromatography; Agilent Technologies, USA) with a high pH RP (reverse phase) column (Durashell, C18, 250mm×4.6mm, 5µm; Bonna-Agela Technologies Inc., USA). Peptides were eluted and combined into 16 groups and lyophilized.

NanoLC-mass spectrometry (MS)/MS analysis

An Ultra 2D Plus nanoflow HPLC (Eksigent Inc., Dublin, CA, USA) coupled with Triple TOF 5600 system (AB Sciex) was used for the analytical separation of peptides^{11,15}. Microfluidic traps and nanofluidic columns packed with ChromXP C18 (3µm, 350µm×0.5mm, Eksigent) were utilized for online trapping and desalting, while nanofluidic columns packed with ChromXP C18 (3µm, 75µm×150mm, Eksigent) were employed in analytical separation. The MS data were acquired in the positive ion mode, with a selected mass range of 350–1500 m/z. Peptides with + 2 to + 5 charge states were selected for MS/MS. MS/MS spectra were acquired in the mass range of 100–1500 m/z. Smart information-dependent acquisition (IDA) was activated with automatic collision energy and automatic MS/MS accumulation.

Database search, iTRAQ quantification and bioinformatics analysis

Protein identification and quantification were performed using ProteinPilot v5.0 (AB Sciex). The quantitative data were loaded to OMICSBEAN¹⁶ website. Data were normalized and t-test was performed. The cutoff value for up-regulation was 1.2-fold change, for down-regulation was 0.83 fold change, and FDR (false discovery rate) p -value < 0.05 was established for significantly differentially expressed proteins (DEPs) between autistic children and controls¹⁷. Principal component analysis (PCA) was performed by using MetaboAnalyst 4.0^{17,18}. Partial Least Squares Discriminant Analysis (PLS-DA) were performed using SIMCA-P 14.1 software package (V14.1, Sartorius Stedim Data Analytics AB, Umea, Sweden). The model was validated using a 7-fold cross validation method and tested with 200 random permutations. The DEPs were loaded to String¹⁹ database for biological process (BP), cellular component (CC), KEGG and REACTOME pathways analysis. Protein-protein interaction (PPI) networks were analyzed using Cytoscape 3.8.2 and STRING.

Metabolomics analysis of plasma

Plasma pretreatment and mass spectrometry analysis

Internal standard (L-2-chlorophenylalanine, 0.3mg/mL, methanol) was pre-mixed with enough methanol-acetonitrile (2:1, v/v) as the metabolite extract, which was pre-cooled at -20°C. For each sample, 100µL plasma was mixed with 300µL methanol-acetonitrile (2:1, v/v), sonicated in an ice water bath for 10 min, stood at -20°C for 30 min, and then centrifugated at 4 °C at 13,000×g for 15 min. Additionally, 100µL supernatant was filled into a glass injection flask, and mixed with 10µL supernatant from each sample as quality control (QC) sample.

A Waters Acquity UPLC system coupled with a Q-TOF Synapt G2 high-definition mass spectrometer (Waters, USA) was used for metabolomics analysis. Chromatographic separation was performed on an ACQUITY UPLC BEH C18 column (2.1×100 mm, 1.7µm) at 45°C. Mobile phase A was an aqueous solution containing 0.1% formic acid, and mobile phase B was an acetonitrile solution containing 0.1% formic acid. The flow rate of mobile phase A and B was 0.4mL/min. The gradient elution is shown in **Table S3**. The injection volume was 2µL. The MS analysis was carried out by the ESI ion source, and the positive and negative ion scanning modes were used to collect the signal respectively. The parameters of MS analysis are shown in **Table S4**. **Processing of the results of metabolomics mass spectrometry**

The raw spectrum was processed by Progenesis QI 2.0 software (Nonlinear Dynamics, Newcastle, UK) for peak selection, comparison, standardization, and identification. R package metaX was used to perform further statistical analysis on the normalized peak intensity. We also used public spectral library MassBank, HMDB, LipidBlast, METLIN for secondary identification. After characterization, duplicate qualitative results are eliminated based on the score.

Metabolomics data analysis

The profile of metabolomics data was analyzed using multivariate statistical methods. PCA and PLS-DA analysis were carried out. VIP (variable importance in projection) score of PLS-DA was obtained. The

metabolites with VIP scores ≥ 1 , fold change ≥ 1.2 or ≤ 0.83 (ASD versus control) as the threshold, and $p < 0.05$ were identified as differential metabolites. Pathway analysis were performed using MetaboAnalyst 4.0.

Integrated analysis of DEPs and differential metabolites identified in this study

The DEPs identified by proteomics analysis between the cases and controls, including the DEPs of plasma, PBMCs, and the differential metabolites between the two groups identified by plasma metabolomics were loaded to MetaScape v3.1.3 in Cytoscape 3.4.0 for integrated analysis²⁰.

Results

The results of gene testing

By genetic testing, among the 122 autistic patients, 5 children were found to be associated with *de novo* mutations in autism risk genes, including histone-lysine N-methyltransferase ASH1L (ASH1L), ATP-dependent RNA helicase DDX3X (DDX3X), N-alpha-acetyltransferase 15, NatA auxiliary subunit (NAA15), GRB10-interacting GYF protein 2 (GIGYF2), and sodium channel protein type 2 subunit alpha (SCN2A), respectively. Another child was found to be associated with the mutations of the MECP2 (methyl-CpG-binding protein 2) gene. Since MECP2 is associated with Rett syndrome, which has been removed from ASD based on DSM-5 diagnostic criteria, so we have only further studied the other five children.

The results of plasma iTRAQ quantitative proteomics analysis

By proteomics analysis, 300 and 350 plasma proteins were identified in the set A experiment and set B experiment, respectively. Among these, 280 proteins were identified as common. The cluster and PCA analysis showed that the expression pattern of plasma proteins was heterogeneous. The characteristics of total plasma protein failed to distinguish ASD individuals from healthy controls (Fig. 2A and B). Out of the total proteins, 13 proteins were identified as DEPs between children with ASD and healthy controls (Fig. 2C, Table S5). Among them, 5 proteins were up-regulated and 8 proteins were down-regulated. The cluster analysis showed that these DEPs could distinguish the ASD group from the control group. However, the five controls were not completely clustered together, and the expression characteristics of the controls with similar age were more similar (Fig. 2D).

The results of PBMCs quantitative proteomics analysis

By proteomics analysis, 2800 proteins were identified as common in the set A experiment and set B experiment. Cluster and PCA analysis showed that the expression characteristics of total proteins failed to distinguish the ASD group from the control group (Fig. 3A and B). By comparative analysis, 111 proteins were identified as DEPs between children with ASD and controls (Fig. 3C, Table S6). Compared

with the controls, 88 proteins were up-regulated and 22 proteins were down-regulated in the ASD individuals. Cluster analysis showed that the ASD group and control group could be well distinguished by these DEPs (Fig. 3D). Of note, among the DEPs identified in PBMCs, the genes encoding 4 DEPs (SLC9A9, CSDE1, CCT4, and DDX3X) are included in the SFARI (Simons Foundation Autism Research Initiative) gene database (<https://gene.sfari.org/>). Interestingly, the DDX3X gene has been detected in gene testing as described above.

Bioinformatics analysis of DEPs in plasma and PBMCs

For the DEPs of plasma, the BP and KEGG pathway are shown in the **Table S7 and S8**. These DEPs were involved in complement activation, immune response, and complement and coagulation cascades (Fig. 4A and B). Interestingly, some complement proteins were related to synapse puncting (C1QA and C1QB) and neuronal development and plasticity (C1QA, C1QB, C1S, C2, and COLEC10).

For the DEPs of PBMCs, the BP, KEGG and Reactome pathways are shown in **Table S9-S11**. These DEPs mainly belong to immunity and metabolism related protein. KEGG pathway showed that they were associated with proteasome, ubiquitin mediated proteolysis, protein processing in endoplasmic reticulum, necroptosis, and nervous system diseases include Parkinson's disease (PD), Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington disease (Fig. 4C-G).

The results of plasma metabolomics analysis

Subsequently, we conducted plasma metabolomics studies with the 5 children with ASD and their corresponding controls. In the positive ion mode of MS analysis, 9281 mass spectrum peaks were identified. The PCA analysis established that the cases and controls could not well be distinguished, although except for a gene, other cases and controls have aggregation trend (Fig. 5A). PLS-DA analysis revealed that the cases and controls could be distinguished (Fig. 5B). However, this model was a bit overfitting (Fig. 5C). It may be due to individual heterogeneity or small sample size. Likewise, 286 metabolites were identified as differential metabolites between the cases and controls (Fig. 5D, **Table S12**). Compared with the controls, 157 of them were increased and 129 were decreased. They could well distinguish the case group from the controls (Fig. 5E), and were mainly related to niacin and niacinamide metabolism, vitamin B6 metabolism, arginine biosynthesis, and sphingolipid metabolism (Fig. 5F). The pathways associated with the differential metabolites are shown in **Table S13**.

In the negative ion mode, 6989 mass spectrum peaks were identified. PCA analysis exhibited that the case group was not well distinguished from the controls. The case group showed a tendency to be more concentrated, while the control group was more dispersed among different individuals (Fig. 6A). PLS-DA analysis separated the two groups effectively, however, this model was also a bit overfitting (Fig. 6B and C). Meanwhile, 190 metabolites were identified as differential metabolites between the cases and the controls. Compared with the controls, 98 were increased and 92 were decreased for controls (Fig. 6D, **Table S14**). They could effectively discern the cases from the control group (Fig. 6E), and were mainly

involved in alanine, aspartic acid and glutamate metabolism, D-glutamine and D-glutamate metabolism, arginine biosynthesis, glycerophospholipid metabolism, and nitrogen metabolism (Fig. 6F, Table S15).

The integration analysis of DEPs and differential metabolites

By integration analysis, two pathways associated with DEPs and differential metabolites identified in this study were enriched, including glycerophospholipid metabolism (Fig. 7A) and N-Glycan biosynthesis (Fig. 7B).

Discussion

In this study, we identified five *de novo* mutational genes associated with five ASD children among 122 clinically diagnosed autistic children. This is similar to results from previous studies of coding-sequence mutations making up 5–10% of ASD patients³. These genes include ASH1L, SCN2A, GIGYF2, NAA15, and DDX3X. They have been reported to be associated with ASD in the previous studies^{21–27}. We then carried out plasma, PBMCs proteomics, and plasma metabolomics studies in these ASD children and healthy controls. The results showed that total plasma and PBMCs proteins, total plasma metabolites, were not well distinguished between the two groups, but DEPs and differential metabolites between these two groups were able to distinguish the two groups. Here, we focus on these DEPs, differential metabolites, and their related mechanisms.

By plasma proteomics analysis, 13 DEPs were identified. Consistent with the previous studies, these DEPs identified in the blood were mainly involved in complement and coagulation cascades, inflammatory and immune responses. Altered complement proteins in the blood from patients with ASD have been broadly reported, including changes in protein expression levels^{15,28–31} and post-translational modifications^{32,33}. Complement C5 was also found to be up-regulated in the PBMCs of children with ASD in our previous study¹¹. Here, C1QA, C1QB, C1S, and C2 were significantly up-regulated in the plasma of children with ASD, whereas COLEC10 was down-regulated. To the best of our knowledge, C1S, C2, and COLEC10 were first reported to be associated with ASD. The expression of most complement proteins is up-regulated in the periphery of autistic patients, suggesting that the complement pathway may be activated in the periphery of autistic patients. However, the expression trends of complement proteins in the brain of autistic patient are not completely consistent^{34,35}. On the other hand, beyond its involvement with innate immune responses, complement proteins has been increasingly implicated in playing an important role in neurodevelopment, including neurogenesis, neuronal migration, and synaptic remodeling^{34–36}. Together, the changes in the complement system in peripheral blood, PBMCs, and brain of ASD patients highlights that this system may play a key role in the pathogenesis of ASD and is worthy of further study.

Accumulating evidence suggests a potential role of the immune system in the pathophysiology of ASD during the pre, neo-, and postnatal periods^{13,37}. In this study, 7 plasma DEPs and 36 PBMCs DEPs were involved in the immune system, further supporting the association between immunity and ASD pathogenesis. Ten DEPs of PBMCs were associated with interleukin-1 (IL-1), together with our previous study (IL-12)¹¹ and other studies^{38,39}, suggesting that IL-1 and IL-12 might be key inflammatory cytokines in peripheral blood and blood mononuclear cells.

Mitochondrial dysfunction has also been implicated in immune dysregulation in ASD⁴⁰. In this study, the expression of three proteins (NDUFA7, NDUFA11, and NDUFA13) related to energy metabolism were increased in PBMCs of autistic children. These results were similar to previous observations in PBMCs¹¹ and LCL cells⁴¹, different tissues and organs⁴², T cell, NK cell, and monocyte⁴³ in children with autism. Activation and proliferation of microglia and astrocytes have also been observed in the brains of ASD subjects⁴⁴. Interestingly, transcriptome analysis between autistic brains and normal brains identified discrete modules by gene co-expression network analysis: a neuronal module and a module enriched for immune genes and glial markers⁴⁵. This may explain the uniqueness of mitochondrial dysfunction in ASD and how autistic children may have nontraditional mitochondrial diseases. After all, mitochondrial dysfunction has been reported by postmortem brain tissue examinations on ASD subjects and reduction of ETC (Electron transport chain) complexes has also been observed in different brain regions of autistic children⁴⁶⁻⁴⁸.

Consistent with our previous study¹¹, the main pathways associated with DEPs of PBMCs have also involved in proteasome, ubiquitin mediated proteolysis, and protein processing in the endoplasmic reticulum, which strongly suggest that endoplasmic reticulum stress (ER) being linked to the PBMCs of children with ASD. ER stress occurs when the amount of unfolded proteins in the ER reach an unmanageable level, triggering the unfolded protein response (UPR)⁴⁹. Under excess or chronic ER stress, cell apoptosis is induced to eliminate unhealthy cells⁵⁰. Here, 6 DEPs were associated with necroptosis. Similarly, LCLs from children with autism were shown to be more sensitive to necrosis than their non-autistic siblings⁵¹. Thus, necroptosis may be associated with the ER stress in the PBMCs of ASD subjects. Moreover, increase in ER stress has been observed in the brains of autistic children^{52,53} and those of ASD model mice⁵⁴. The mutation of ASD related genes and the aggregation of their encoded proteins^{11,52} and oxidative stress may be responsible for ER stress in children with ASD⁵². On the other hand, the proteostasis network machinery plays a role in the establishment, maintenance, and plasticity of stable and dynamic dendritic arbors. Ubiquitin-proteasome system is required for developmental dendritic pruning^{55,56}.

Interestingly, among the DEPs of PBMS, three other genes have also been linked to ASD in addition to DDX3X, including SLC9A9⁵⁷, CSDE1⁵⁸, and CCT4⁵⁹. Among them, The SLC9A9 gene encodes Na⁺ /H⁺ transporter-9 endometrium protein (NHE9), which has been shown to be related to endocytosis, protein ubiquitination, and phagosome⁵⁷. CSDE1 encodes RNA-binding proteins that may be involved in

translation-coupled mRNA conversion and is associated with neurodevelopment and neuropsychiatric disorders⁵⁸. The CCT4 gene encodes a molecular chaperone that assists in protein folding during ATP hydrolysis⁶⁰. These results further support that protein folding and ER stress may be associated with the pathogenesis of ASD, and that at least some children with ASD carry more than two risk genes in this study.

By metabolomics analysis, the differential metabolites in the plasma between cases and controls were mainly involved in amino acid (alanine, aspartate and glutamate, arginine biosynthesis, and D-glutamine and D-glutamate), vitamin (nicotinate and nicotinamide, and vitamin B6), and lipid (glycerophospholipid and sphingolipid) metabolism. Nitrogen metabolism, N-Glycan biosynthesis, and neomycin, kanamycin and gentamicin biosynthesis were also involved.

In the present study, among the altered amino acids, L-Glutamate was down-regulated in plasma of controls while L-Glutamine was increased in plasma of autistic children. Although previous studies showed that the altered levels of glutamate and glutamine in the blood of autistic patients were inconsistent⁶¹⁻⁶⁶, these data support the current view that excitatory/inhibitory imbalance, especially the abnormality of the excitatory neurotransmitter glutamate, is one of the pathogenesis of ASD^{65,67}. Indeed, it has been reported that glutamate signals in the anterior cingulate cortex and cerebellum of ASD patients were significantly decreased⁶⁸. More recently, the gut metabolites involved in alanine, aspartate, and glutamate metabolic pathways were reported to be significantly lower in children with ASD, which was associated with differences in the abundance of gut microbiota related to D-Glutamine and D-glutamate metabolism, suggesting that the gut microbiota might contribute to abnormal glutamate metabolism in autistic children⁶⁹. Besides, similar with the present study, aspartate has been observed to be decreased in the fecal ASD subjects⁷⁰, while ornithine was observed to be increased in the blood of children with ASD⁶⁴.

Our results showed that three differential metabolites were also involved in the nicotinate and nicotinamide metabolism pathway. Niacin (NA), also known as Vitamin B3 and nicotinic acid, can be biosynthetically converted into nicotinamide adenine dinucleotide (NAD). NAD has a variety of biological functions and plays a central role in redox reactions⁷¹. Nicotinate and nicotinamide metabolism have been reported to be altered in the prefrontal cortex⁷², urine⁷³, and blood⁷⁴ of ASD individuals, and associated with microbiota transfer therapy (MTT) of autistic children⁷⁴. Nicotinamide is derived from tryptophan, while abnormal tryptophan metabolism has been observed in children with ASD^{73,75-77}. In addition, three differential metabolites (O-Phospho-4-hydroxy-L-threonine, Pyridoxamine, and 4-Pyridoxate) involved in Vitamin B6 metabolism were found to be decreased in autistic children. Reduced levels of Vitamin B6 have been observed in the urine of autistic children⁷⁸. It is the main cofactor of biological reactions and is important for the synthesis of neurotransmitters and trans-sulfuration. Its deficiency is related to oxidative stress, high blood homocysteine and hypomethylation in children with AD⁷⁸. In general, the lack of vitamin B group in children with ASD may be caused by nutritional deficiency, poor absorption or alteration in gut microbiota^{74,79,80}, contributing to the pathogenesis of ASD.

The brain is particularly enriched in lipids with a diverse lipid composition compared to other tissues⁸¹. Glycerophospholipids are critical components of neuronal membranes and myelin, and principal regulators of synaptic function⁸¹. Sphingolipid is involved in neuronal differentiation, synaptic transmission in neuronal-glia connections, and myelin stability. Disturbance of their metabolism has been linked to various neuropsychiatric diseases include autism⁸², Rett syndrome⁸³, and ASD⁸⁴.

Finally, integrating omics data, glycerophospholipid and N-linked glycosylation metabolism was associated with the DEPs and differential metabolites. N-linked glycosylation is important in brain structure and function. The extracellular glycans and glycoconjugates may contribute to the etiology and pathogenesis of pervasive neurodevelopmental disorders include idiopathic ASDs. Glycobiology related genes were implicated in ASD. Mutations in glyco genes associated with ASD affect the downstream steps of N-glycan biosynthesis⁸⁵.

Limitations

Although we detected 122 children in the genetic testing, and the rate of children carrying risk genes is also consistent with the previous report³, the rate itself is low. Thus, the number of samples carrying risk genes used in omics studies is limited, and further research with a large sample size is required. Secondly, in further research, it is also interesting to add a group in which that the children were diagnosed as ASD, but not to be detected to carry risk gene.

Conclusions

In this study, our results showed that although there was heterogeneity at the genetic level in children with ASD, the DEPs of plasma and PBMCs and differential metabolites of plasma could still distinguish the cases from controls. The proteomic results highlighted the roles of complement, inflammation and immunity, mitochondrial dysfunction, proteasome, ubiquitin mediated proteolysis, and ER stress in the pathogenesis of ASD. Metabolomic results mainly showed the disturbances of amino acid, vitamin, and lipid metabolism in children with ASD (Fig. 8). The mechanisms and pathways associated with the DEPs and differential metabolites have been reported in previous studies. The results agreed with the view that children with ASD might have an important underlying common mechanism. They are not only potential therapeutic targets for ASD but also significant contributors for studying biomarkers for the disorder^{1,6-8,13}.

Abbreviations

ASD
autism spectrum disorder
BP
biological process
CC

cellular component
DEPs
differentially expressed proteins
ER
endoplasmic reticulum stress
iTRAQ
isobaric tagging reagents for relative and absolute quantification
MS
mass spectrometry
PLS-DA
orthogonal projections to latent structures-discriminant analysis
PBMCs
peripheral blood mononuclear cells
PCA
principal component analysis
VIP
variable importance in projection.

Declarations

Acknowledgments

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Authors' contributions

LMS and CYF. conceived and designed the experiments; HJZ, JL, XKL, YXZ, and XXT performed the experiments and analyzed the data; LMS and XSC wrote the manuscript. MC, NUK, and XSC reviewed and edited the manuscript. YG, CYF, and QH carried out the clinical studies and collected the blood samples. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The Maternal and Child Health Hospital of Baoan, and the Shenzhen University approved study procedures. The experiments were performed after obtaining written consent from caretakers of the children under observation according to the guidelines of this hospital.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Figures

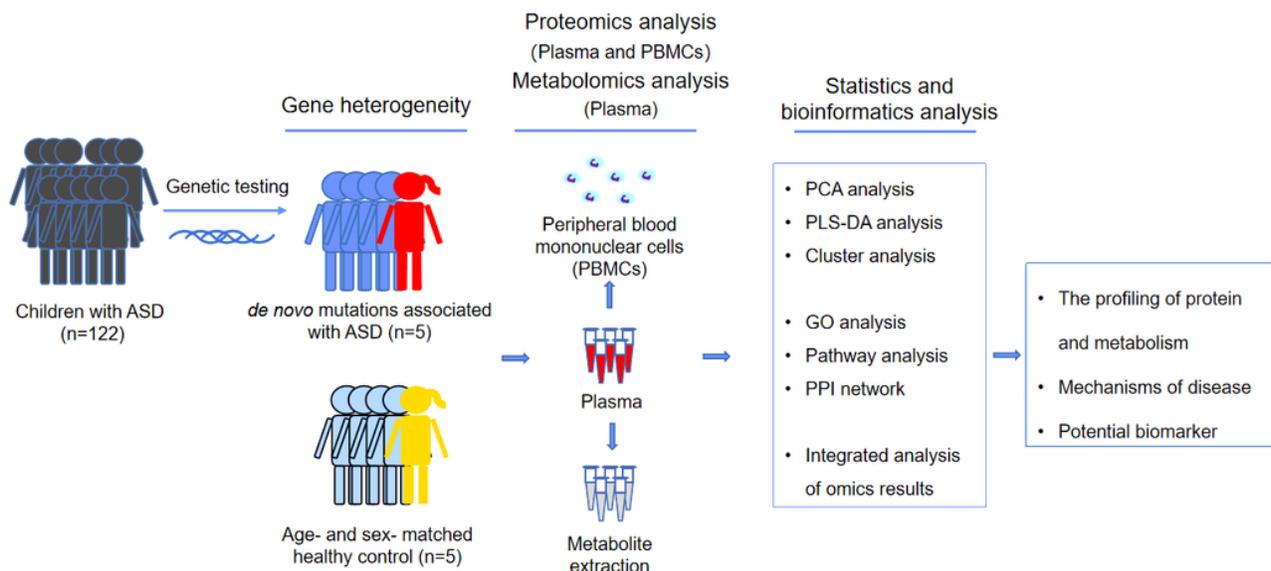


Figure 1

Flow diagram of the study. An overview of the workflow used in this study is shown in Figure 1.

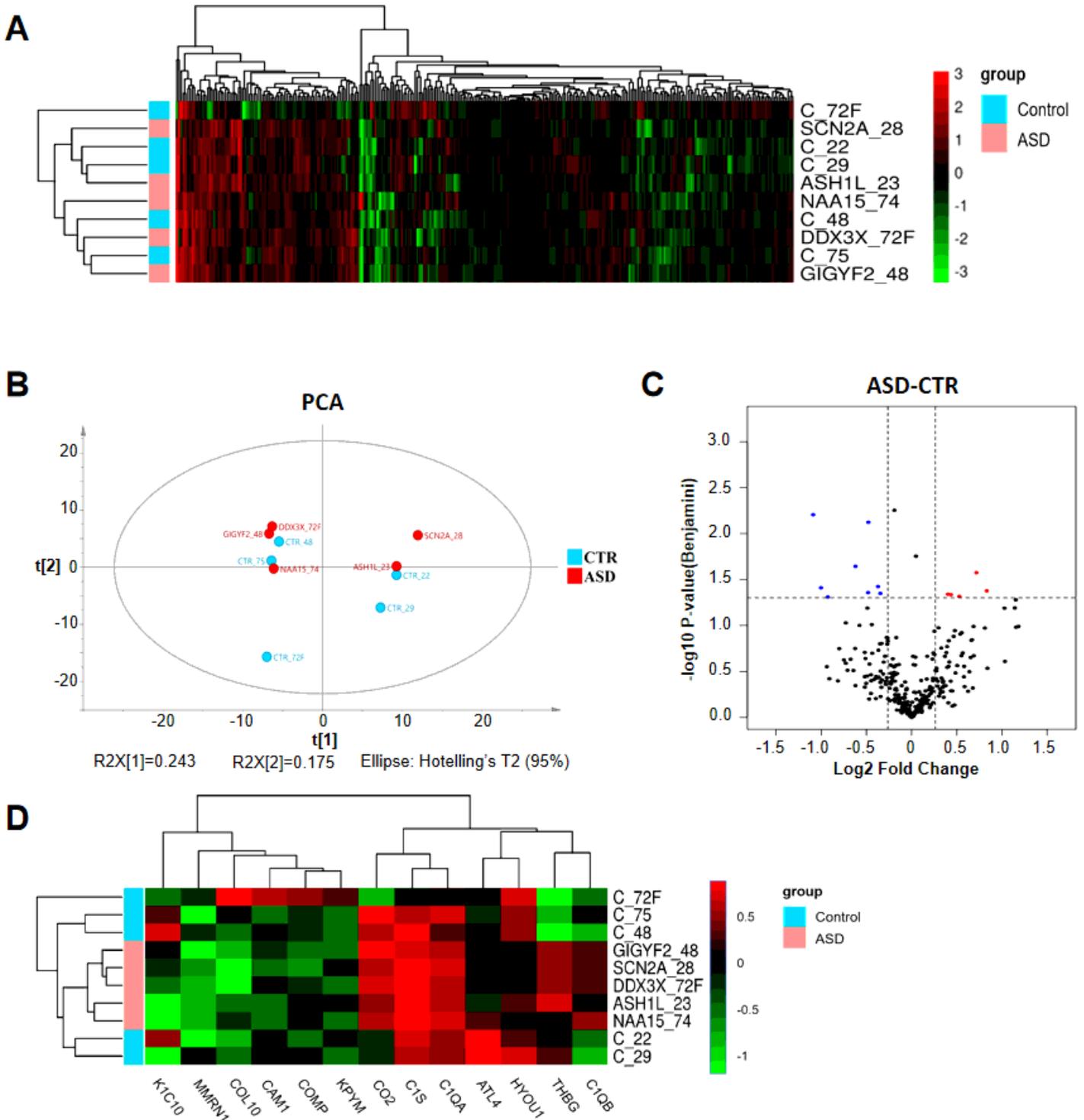


Figure 2

The DEPs identified in plasma between children with ASD and healthy controls. (A) Cluster map of plasma protein expression in the children with ASD and healthy controls. (B) PCA analysis of the plasma protein expression in children with ASD and healthy controls. (C) Volcano plots depicted the distribution of plasma proteins between children with ASD and healthy controls. The log2 fold change (FC) is plotted

versus the $-\log_{10}$ of the P value (Benjamini). Red dots = hits with $p < 0.05$ and mean $\log_2FC > 0.26$; blue dots=hits with $p < 0.05$ and mean $|\log_2FC| < 0.26$. (D) Cluster analysis of the DEPs of plasma.

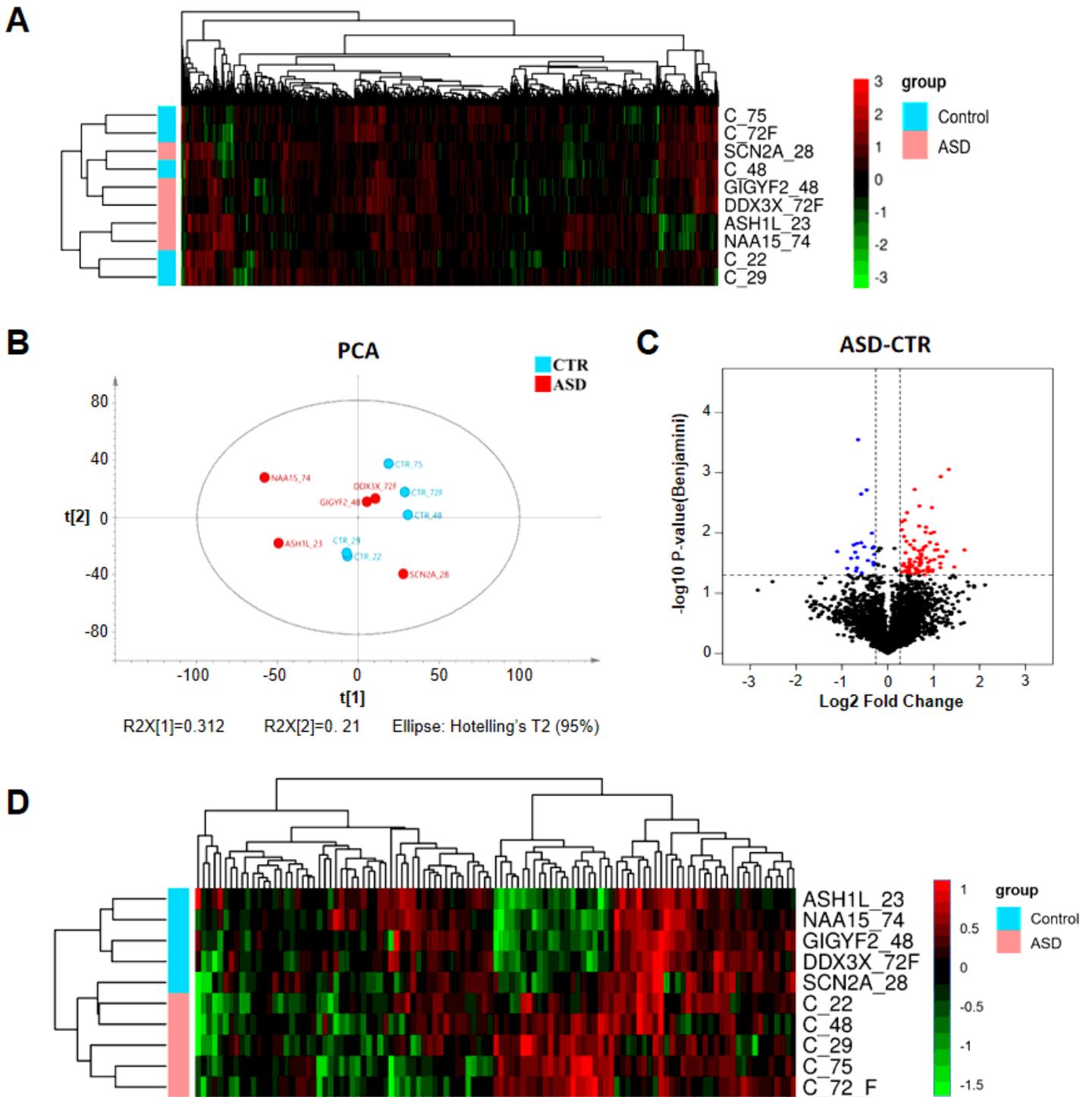


Figure 3

The DEPs identified in PBMCs between children with ASD and healthy controls. (A) Cluster map of PBMCs protein expression in the children with ASD and healthy controls. (B) PCA analysis the PBMCs protein expression in children with ASD and healthy controls. (C) Volcano plots depicted the distribution

of PBMCs proteins between children with ASD and healthy controls. The log2 fold change (FC) is plotted versus the $-\log_{10}$ of the p-value (Benjamini). Red dots = hits with $p < 0.05$ and mean $\log_2FC > 0.26$; blue dots = hits with $p < 0.05$ and mean $|\log_2FC| < 0.26$. (D) Cluster analysis the DEPs of PBMCs.

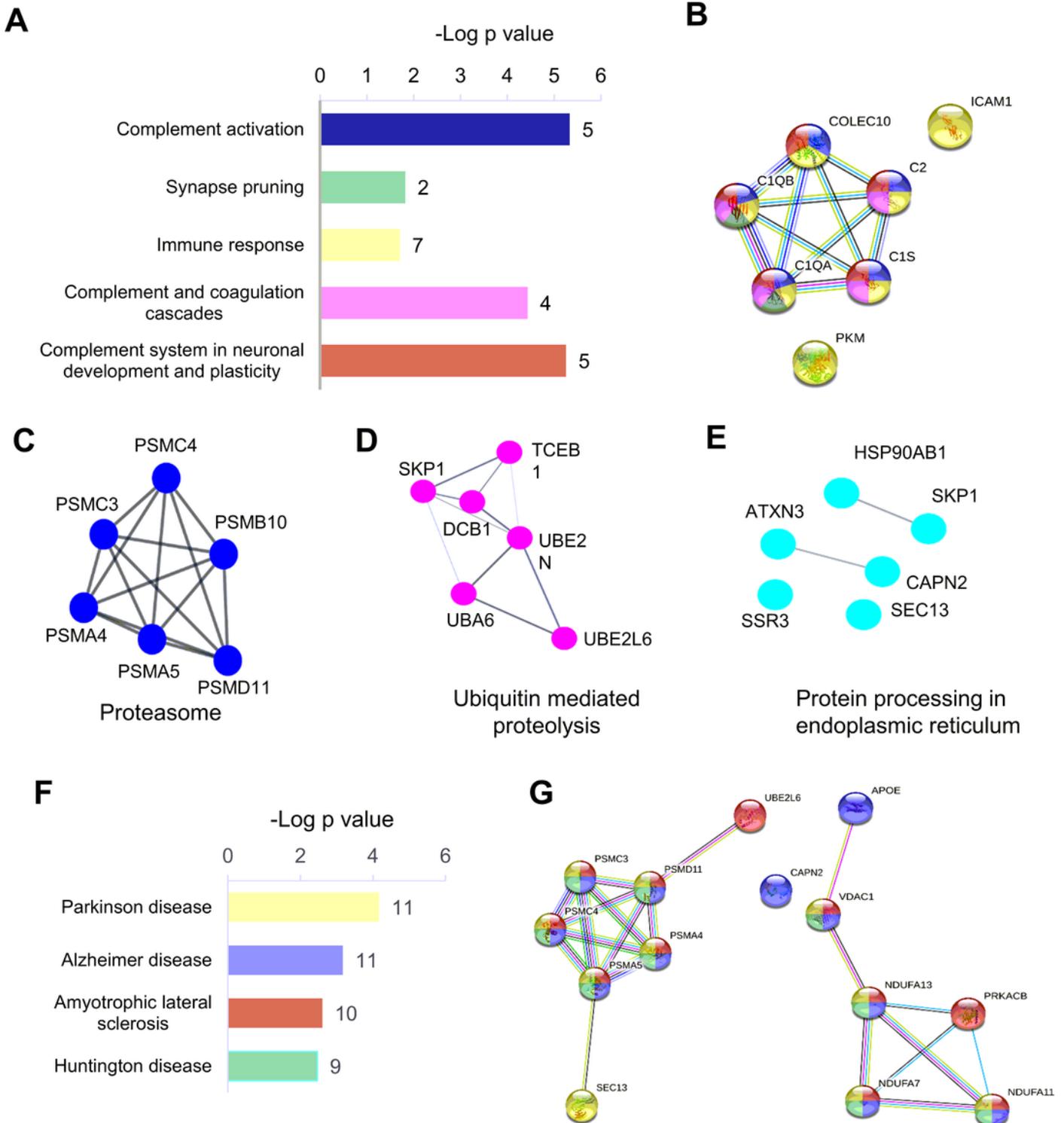


Figure 4

Bioinformatics analysis of the DEPs of plasma and PBMCs between children with ASD and healthy controls. (A) The key biological process (BP) associated with DEPs of plasma. Number of proteins

associated with each category is shown on the right of each term bar. (B) PPI network linked to the plasma DEPs. The BP shown by the color of the bar chart in A is consistent with that shown by the same color in B. (C)-(E). The key KEGG pathways associated with DEPs of PBMCs. (F) The DEPs of PBMCs linked to the KEGG pathways of neurological diseases. Number of proteins associated with each category is shown on the right of each term bar. (G) PPI network linked to the DEPs of PBMCs associated with the KEGG pathways of neurological diseases. The pathways represented by the same color in F and G are consistent.

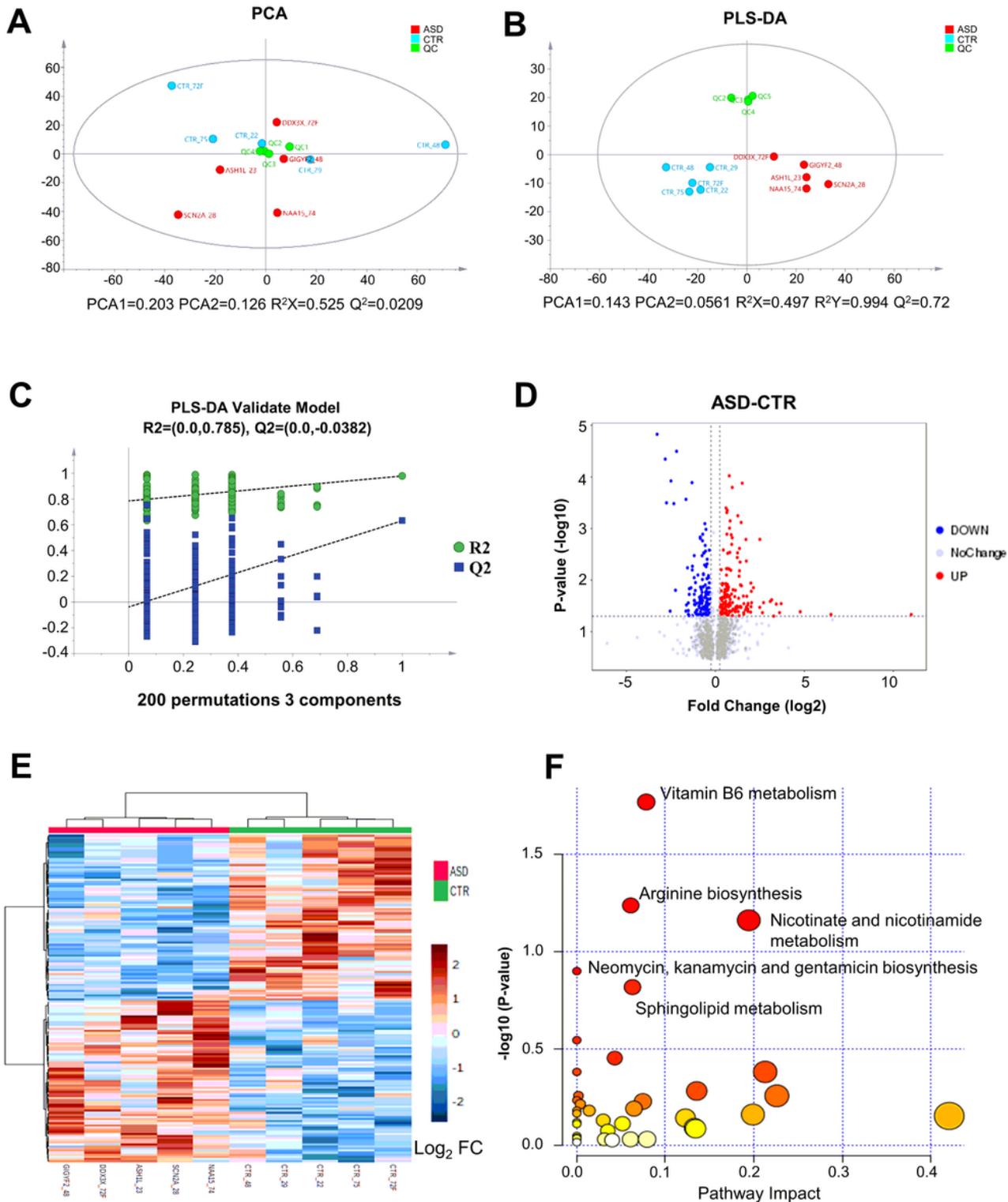


Figure 5

Statistical and bioinformatics analysis of metabolites detected by mass spectrometry in positive ion mode. (A) PCA analysis the metabolites of the case and the control group. (CTR). (B) PLS-DA analysis the metabolites of the cases and the control group. (C) PLS-DA model was verified by permutation test. (D) Volcano plot analysis of differential metabolites between children with ASD and healthy controls. The \log_2 fold change (FC) is plotted versus the $-\log_{10}$ of the p value. Blue and red dots: hits with $p < 0.05$ and $|\log_2\text{FC}| > 0.26$. (E) Cluster analysis the differential metabolites between children with ASD and healthy controls. (F) Metabolic pathways related to differential metabolites between children with ASD and healthy controls.

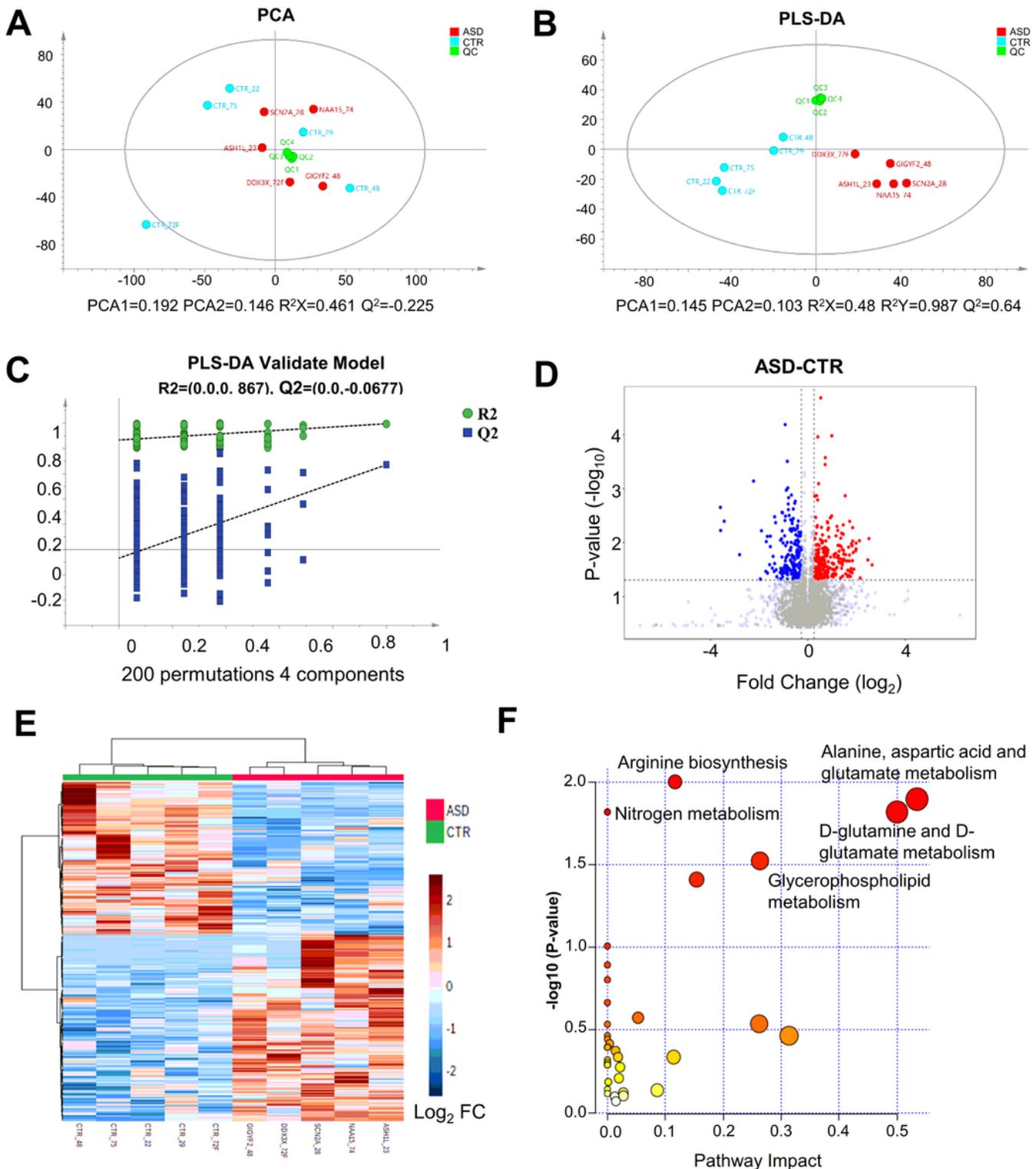


Figure 6

Statistical and bioinformatics analysis of metabolites detected by mass spectrometry in negative ion mode. (A) PCA analysis the metabolites of the case and the control group. (B) PLS-DA analysis the metabolites of the cases and the control group. (C) PLS-DA model was verified by permutation test. (D) Volcano plot analysis of differential metabolites between children with ASD and healthy controls. The \log_2 fold change (FC) is plotted versus the $-\log_{10}$ of the p value. Blue and red dots: hits with $p < 0.05$ and

$|\log_2FC| > 0.26$. (E) Cluster analysis the differential metabolites between children with ASD and healthy controls. (F) Metabolic pathways related to differential metabolites between children with ASD and healthy controls.

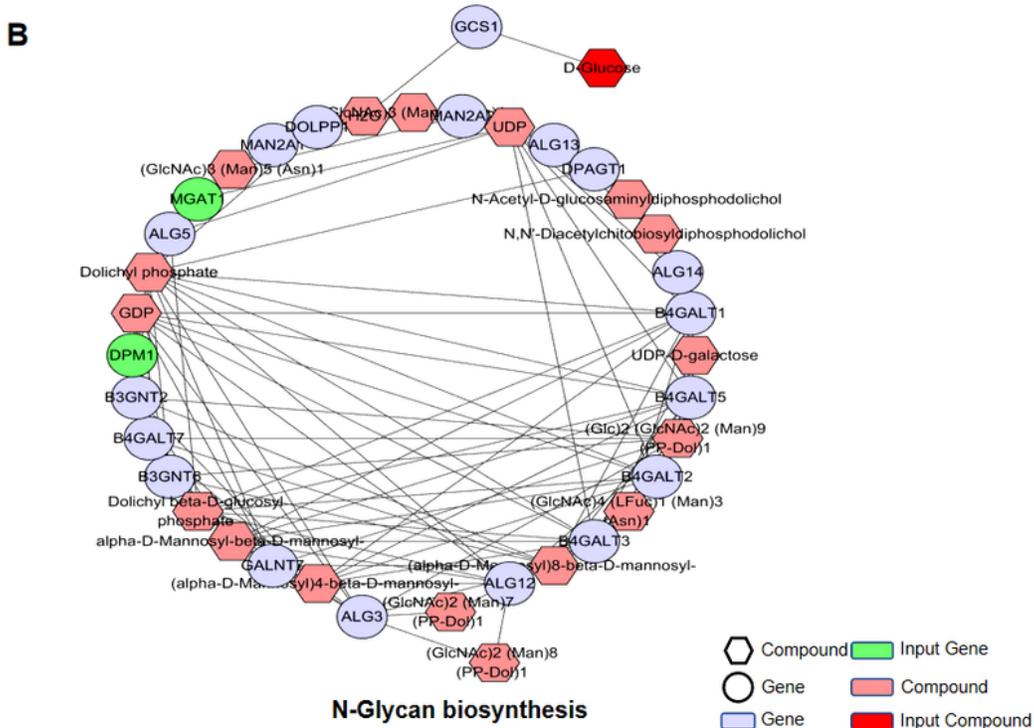
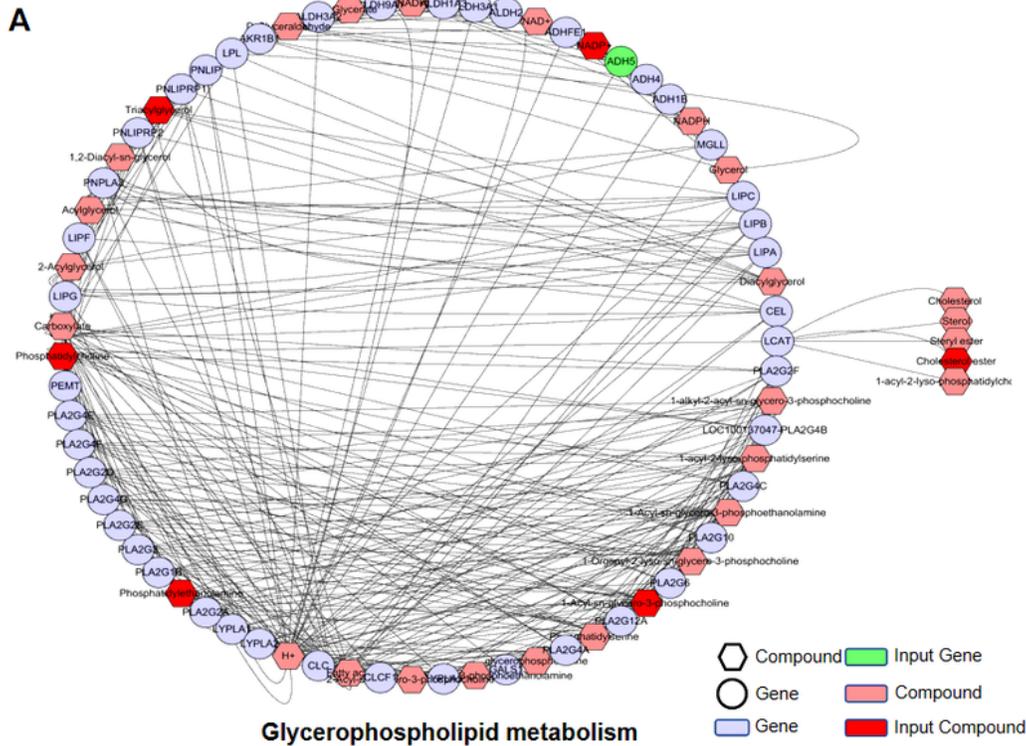


Figure 7

Integrated analysis of proteomics and metabolomics results. (A) Glycerophospholipid metabolism was associated with the DEPs of proteomics and the differential metabolites of metabolomics. (B) N-Glycan

biosynthesis was associated with the DEPs of proteomics and the differential metabolites of metabolomics.

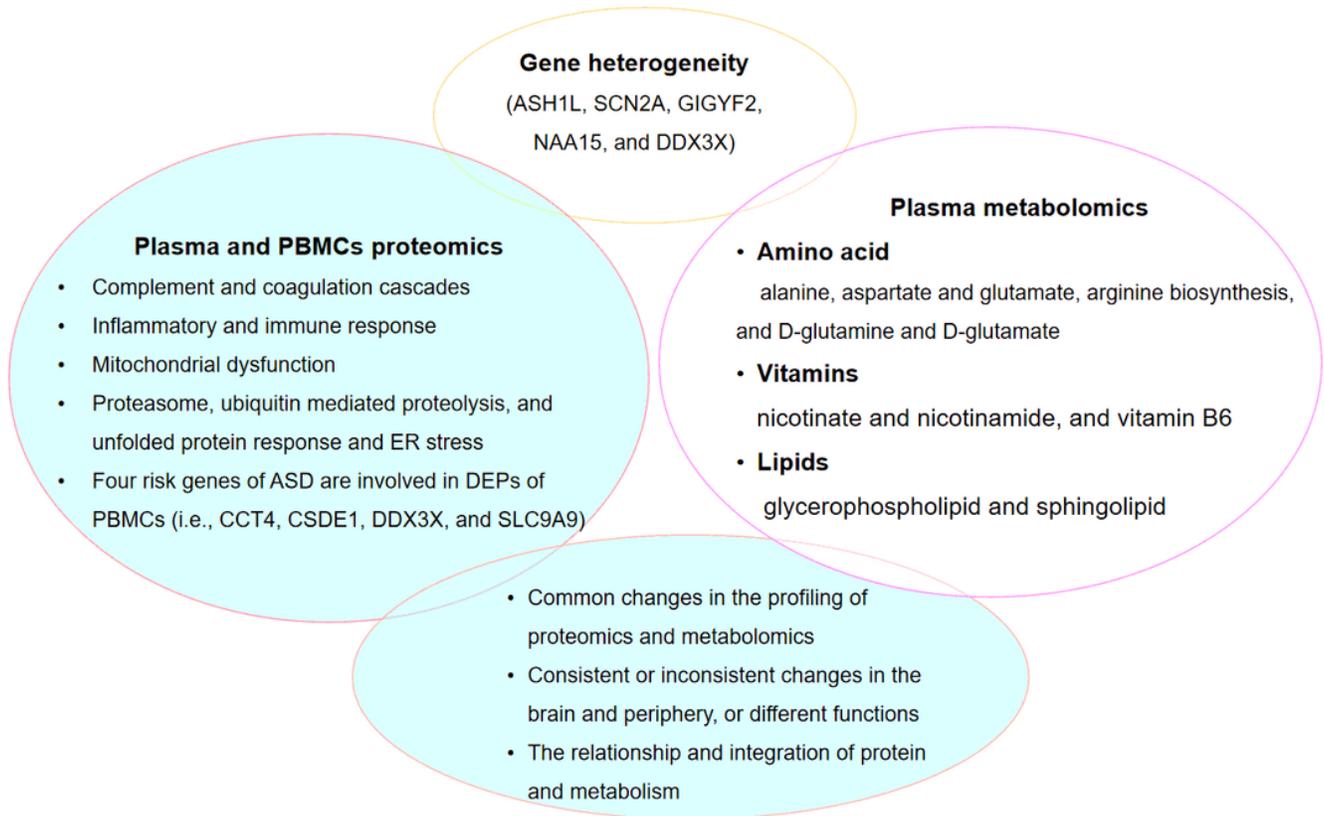


Figure 8

Common mechanisms and potential biomarkers can be obtained in children with ASD with different risk gene. Bold letters indicate that these pathways are related to the DEPs and differential metabolites in this study.

Supplementary Files

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