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iTRAQ-Based Proteomic Analysis Reveals Protein Profile in Plasma from Children with Autism

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Purpose: **Autism is a childhood neurological disorder with poorly understood etiology and pathology. This study is designed to identify differentially expressed proteins that might serve as potential biomarkers for autism.**
Experimental design: **We perform iTRAQ (isobaric tags for relative and absolute quantitation) analysis for normal and autistic children's plasma of the same age group.**
Results: **The results show that 24 differentially expressed proteins were identified between autistic subjects and controls. For the first time, differential expression of complement C5 (C5) and fermitin family homolog 3 (FERMT3) are related to autism. Five proteins, that is, complement C3 (C3), C5, integrin alpha-IIb (ITGA2B), talin-1 (TLN1), and vitamin D-binding protein (GC) were validated via enzyme-linked immunosorbent assay (ELISA). By ROC (receiver operating characteristic) analysis, combinations of these five proteins C3, C5, GC, ITGA2B, and TLN1 distinguished autistic children from healthy controls with a high AUC (area under the ROC curve) value (0.982, 95% CI, 0.957–1.000, $p < 0.000$).**
Conclusion: **These above described proteins are found involved in different pathways that have previously been linked to the pathophysiology of autism spectrum disorders (ASDs). The results strongly support that focal adhesions, acting cytoskeleton, cell adhesion, motility and migration, synaptogenesis, and complement system are involved in the pathogenesis of autism, and highlight the important role of platelet function in the pathophysiology of autism.**

1. Introduction

Autism spectrum disorder (ASD) is characterized by impaired social interactions and communications, with restricted interests.[1] ASD includes classical autism, pervasive developmental disorder not otherwise specified (PDD-NOS), and Asperger's syndrome (AS). Among them, autism is the most severe and can be distinguished from AS and PDD-NOS by a delay in language development and severity of behavioral and intellectual impairments.[2] Its onset occurs during the first 3 years childhood and there is a male preponderance, with a sex ratio of 4:1.[3] The prevalence of ASD has been steadily increasing for several decades and now is approaching up to 1% in the human population, which can be a heavy burden for the family of affected children.[3]

Although the exact causes of ASD are still unknown, previous studies suggest that autism can be considered a multifactorial disease in which both genetic and environmental factors are involved.[4] Meanwhile, as the etiology of autism is not completely understood, there is still no medication available that can be used for treatment of autism. However, some behavioral treatments are available to improve core and associated symptoms of autism, particularly when initiated at an early stage.[5] Thus, there is an increasing demand for finding biomarkers of autism as they could help to identify children with autism as early as possible. Regretfully, so far, no biomarkers are yet reported for early diagnosis or prediction of autism. The diagnosis of autism is often delayed, which translates into a missed opportunity to provide treatment during a critical developmental period.[6] Even more, a recent report showed that a typical autism tended to be diagnosed more often at an age of 5–6 years.[7] In addition, diagnosis of autism is based on neuropsychological tests, the patient's history, and the observation of behavioral abnormalities, including language delays. These diagnostic measures can be considered arbitrary or subjective and may generate many false positives. Consequently, it is need of the hour to search for an objective biomarker for early diagnosis of autism.

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Clinical Relevance

Currently, there is little knowledge about the potential biomarkers of autism, which might be used as an authentic laboratory test for the diagnosis of autism. The present study was designed to find some potential plasma biomarker proteins that may serve as diagnostic tools, which was the first report to identify the differentially expressed proteins in the plasma of autistic children compared to healthy controls by using iTRAQ (isobaric tags for relative and absolute quantitation) technique. Twenty-four differentially expressed proteins were identified in this study. Most of them were found involved in focal adhesions, acting cytoskeleton, cell adhesion, motility and migration, synaptogenesis, complement system, and platelet function. Moreover, these findings support current views on the molecular mechanism of ASD and highlight the important role of platelet function in the pathophysiology of autism. For the first time, two proteins, that is, C5 and FERMT3 were associated with autism. The panel of five candidate proteins (C3, C5, GC, ITGA2B, and TLN1) could distinguish autistic childes from healthy controls with a high AUC value. Hence, these might prove as diagnostic biomarkers in future studies.

Multiple approaches are being used to discover new biomarker panels for ASD. The most commonly employed techniques to detect ASD susceptibility genes is the whole-exome sequencing, the chromosomal microarray, and the selective candidate gene analysis.[2] Numerous autism candidate genes have been reported.[8] However, none of these provided adequate specificity or accuracy to be used in ASD diagnosis, limiting the current utility of genetic testing for ASD.[8] On the other hand, many different variants converge on common biological pathways, indicating that etiological heterogeneity, variable penetrance, and genetic pleiotropy are pervasive characteristics of autism. At present, highly penetrant mutations leading to ASD have been described, with proved clinical utility.[9] Likewise, recent advances in defining the molecular and cellular pathology of ASD suggested that altered patterns of neuronal connectivity in the developing brain can be considered as the neurobiological basis of these disorders.[10] Functional magnetic resonance imaging studies have demonstrated altered patterns of cortical activation in diagnosed children and adults with ASD during the performance of social and cognitive tasks, as well as abnormal intrinsic functional connectivity.[11] However, imaging examination is clinically impractical for high-throughput population-based screening.

Plasma proteome analysis provides a potentially promising approach in searching the disease biomarkers. However, in past years, limited studies are reported on proteomics based research on autism. Thus, in order to discover potential protein biomarkers with diagnostic utility and to further clarify the molecular mechanism in autism, we carried out iTRAQ-based (where iTRAQ is isobaric tags for relative and absolute quantitation) plasma analysis to compare protein profiles from autistic and healthy control children in this study.

2. Experimental Section

2.1. Plasma Samples

In this study, 24 male and 6 female autistic patients (2–6 years old) were taken from Populations and Family Planning Hospital of Baoan and subjected for comparative autism analysis. They were matched one case with one control based on age and gender (Supporting Information, Table 1). The autism was diagnosed by a child neuropsychiatrist based on the criteria of autistic disorders as defined in the *Diagnostic and Statistical Manual of Mental Disorder—Fourth Edition* (*DSM-IV*). There were no significant differences in the distributions of weight, height, or body mass index (BMI) between the case and control groups. The experiments were conducted with the written consent of the caretakers of the children under observation according to the guidelines of the Human Research Ethics Committees of Populations and Family Planning Hospital of Baoan. Blood samples (5 mL) were collected in EDTA-coated plastic tubes in the morning and in the fasting state, and centrifuged at 3000 × *g* for 10 min at room temperature. The supernatants were then divided and stored in aliquots at −80 °C for further analysis.

2.2. Protein Enrichment

Plasma samples were analyzed in three groups in the iTRAQ analysis. In each group, equal amounts of plasma from five autistic and five age- and sex-matched healthy controls were pooled (the ratio of male to female is 4:1, Supporting Information, Table 1), respectively. The pooled plasma samples were pretreated with Proteo-Miner™ protein enrichment kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. To verify the efficiency of pretreatment, crude plasma (without depletion) and eluted fractions were separated on SDS-PAGE followed by Coomassie Brilliant Blue staining R-250.

2.3. iTRAQ Labeling and High-pH RPLC Fractionation

The iTRAQ analysis was performed according to the previous study.[12,13] Plasma proteins (100 μg) were reduced, alkylated, and digested with trypsin (Promega, Madison, WI, USA) at a ratio of 1:30 at 37 °C for overnight, and then labeled with the iTRAQ reagents (AB Sciex, Foster City, CA, USA). Three samples from autistic children were labeled with iTRAQ tags 113, 114, 115, and the healthy controls samples were labeled with tags 116, 117, and 118, respectively. After labeling, the samples were incubated at room temperature for 1 h, and then the labeled samples were mixed and lyophilized.

The dried samples were reconstituted in 100 μL double-distilled water and injected into the Agilent HPLC (HPLC; Agilent Technologies, Santa Clara, CA, USA) with a high pH RP column (Durashell, C18, 250 mm × 4.6 mm, 5 μm; Bonna-Agela Technologies, Inc., Wilmington, DE, USA). Peptides were eluted and combined into 10 groups and lyophilized and stored at −80 °C.[12,13]

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2.4. NanoLC-MS/MS (Mass Spectrometry) Analysis

An Ultra 2D Plus nanoflow HPLC (Eksigent, Inc., Dublin, CA, USA) coupled with Triple TOF 5600 system (AB Sciex) was used for analytical separation of peptides.[12,13] Microfluidic traps and nanofluidic columns packed with ChromXP C18 (3 μm, 2.1 × 100 mm, Eksigent) were utilized for online trapping and desalting, while nanofluidic columns packed with ChromXP C18 (3 μm × 150 cm, Eksigent) were employed in analytical separation. The mass spectrometer data 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 400–1250 m/z. Smart information-dependent acquisition (IDA) was activated with automatic collision energy and automatic MS/MS accumulation.[12,13]

2.5. Database Search and iTRAQ Quantification

The protein identification and quantification were performed using ProteinPilot v4.5 (AB Sciex) with the Paragon Algorithm against the UniProt "complete proteome" human proteins database. Based on 95% confidence level, at least one unique peptide per protein group was required for identifying proteins, and two quantified peptides were required for quantifying protein. The cutoff values of 1.5-fold for upregulated proteins and of 0.67-fold for downregulated proteins with p-value <0.05 were established as differentially expressed proteins between autistic patients and controls. The changes with EF (error factor) greater than 2 and/or these changed proteins that were not consistent in direction among three groups were excluded. The EF as a measure of the error in the average ratio expressing the 95% confidence interval (95% CI) of the average iTRAQ ratio (EF = $10^{95\%\ \mathrm{CI}}$, where 95% CI = (ratio × EF) − (ratio/EF)).[14] The total proteins were compared with plasma proteome database (http://plasmaproteomedatabase.org/). Hierarchical cluster analysis was performed by using Cluster 3.0 software (Michael Eisen, Stanford University, CA, USA).

2.6. Bioinformatics Analysis

The full set of dysregulated proteins was entered in DAVID (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov/) database for functional analysis. Functional interaction network analysis was carried out by using ClueGO cytoscape plugin.[15] Gene ontology (GO) categories and pathways searched include biological processes, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, REACTOME pathway, and Wiki pathway. The protein–protein interaction (PPI) networks associated with these proteins were retrieved from a web-based tool LENS (Lens for Enrichment and Network Studies of Proteins) at the website: http://severus.dbmi.pitt.edu/LENS/.[16]

2.7. Validation of Protein Expression by ELISA Analysis

To validate quantitative proteomic results, plasma C3, C5, ITGA2B, GC, and TLN1 concentrations in individual samples from autistic and healthy controls were measured using commercial ELISA kits (USCN Life Science, Inc., Cloud-Clone Corp., USA), according to the manufacturer's instructions. Twenty nine (29) aqueous crude plasma samples were used from both groups for ELISA analysis (Supporting Information, Table 1). The ELISA results were normalized to total protein concentration.

2.8. Statistical Analysis

The data are presented as mean ± standard deviation (SD) and statistical analyses were performed by two-tailed t-test. P-value < 0.05 was considered statistically significant. ROC curves were constructed to assess the diagnostic values of the candidate proteins by using SPSS software 19.0 (IBM Analytics, New York, NY, USA).

3. Results

3.1. Enrichment of Medium and Low Abundance Plasma Proteins

The most challenging obstacle to develop blood-based biomarkers is the massive dynamic range of proteins in blood. Here, we used Proteo-Miner^{TM} enrichment kit to enrich medium and low abundant plasma proteins. The protein patterns of plasma samples before and after enrichment were visualized on SDS-PAGE gels (**Figure 1A**). As expected, by the pretreatment, the low and medium abundance proteins could be enriched.

3.2. iTRAQ Comparative Proteomics Results

By iTRAQ analysis, 625 plasma proteins were successfully identified (Supporting Information, Table 2), out of which 45, 75, and 85 proteins were identified as differentially expressed proteins between autistic patients and healthy controls in group1, group2, and group3, respectively. Among the three groups, 24 differentially expressed proteins were found common (**Table 1**). The hierarchical cluster analysis showed that the total proteins expression pattern in the three groups was similar (Figure 1B). By comparison with plasma proteome database, as shown in Figure 1C, 542 proteins identified in this study overlapped with the proteome database and 83 proteins have no hit in the database. Interestingly, all of the 24 differentially expressed proteins matched with the database (Supporting Information, Table 2). The cluster map of differentially expressed proteins identified common in different groups is shown in Figure 1D.

Among the 24 proteins identified common in the three groups, 10 proteins were significantly upregulated and 14 downregulated (Table 1). Out of these 24 proteins, 22 proteins have already been reported to be associated with autism (Table 1). For the first time, differential expression of 17 proteins, that is, ACTN1, ENO1, PARVB, CALM1, CALR, C5, EHD3, FERMT3, FBLN1, IGFALS, ITGA2B, MAPRE2, TLN1, THBS1, VCP, VCL, and VTN are observed in the blood/plasma of autistic patients compared with the healthy controls. Two proteins, that is, C5 and FERMT3 are being reported for the first time to be associated with autism. Likewise, we performed the FDR (false discovery rate) correction for

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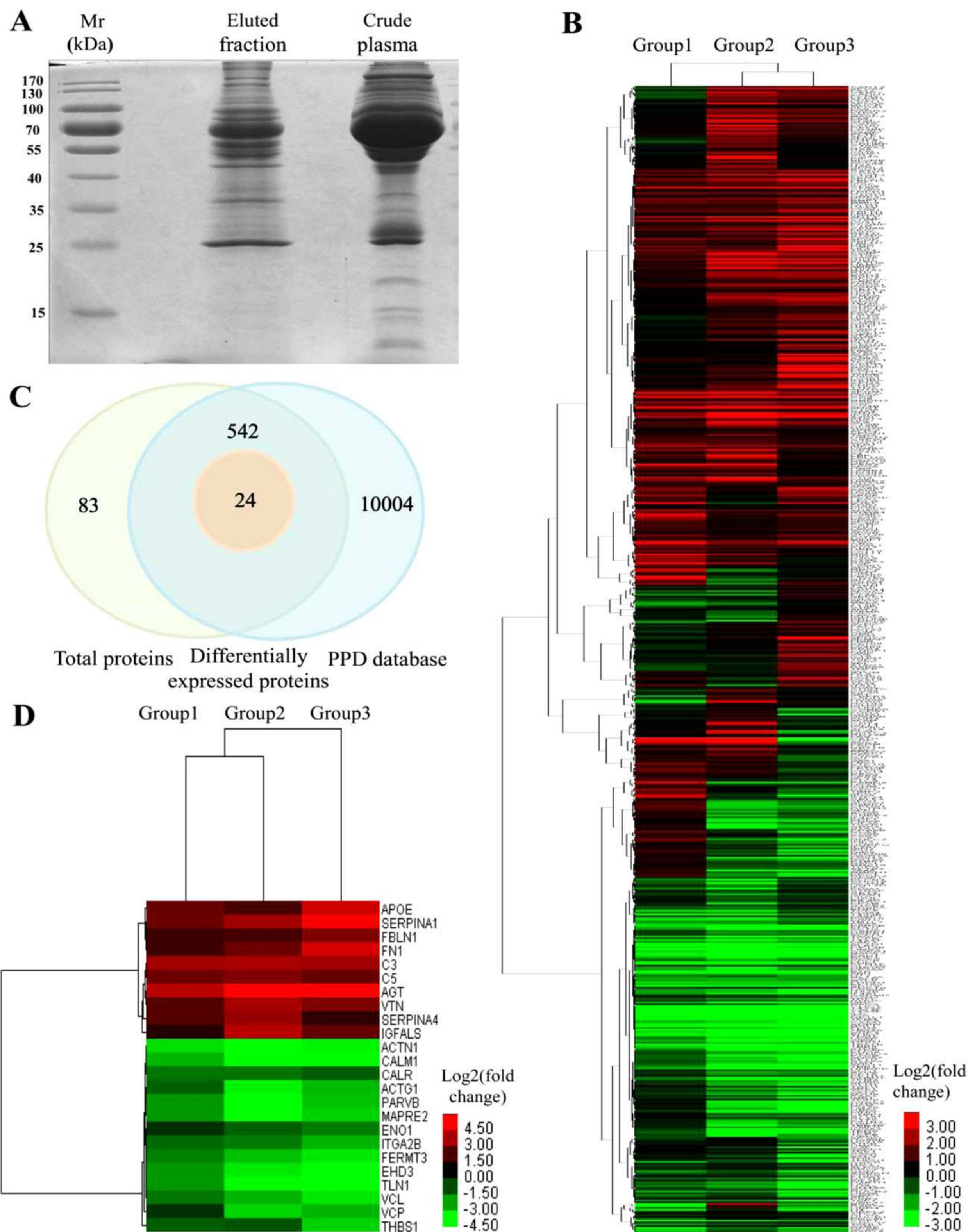

Figure 1. The efficiency of enriched plasma proteins and venn diagram of proteins. A) The efficiency of enriched plasma proteins. Twenty microgram crude plasma (without depletion) and eluted fractions (enrichment plasma proteins) were separated on SDS-PAGE gel. Mr, Molecular weight. B) Cluster map comparing the protein expression patterns of different groups. Red color indicates higher expression, green lower expression, and black indicates the same expression levels compared with the control. C) Venn diagram showing the overlapping of proteins identified in this study and plasma proteome database. D) Cluster map comparing the differentially expressed proteins found common in different groups.

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Table 1. Differentially expressed plasma proteins in autistic and age- and sex-matched healthy controls.

Protein name	Accession no.	Gene name	Peptides (95%)	% Cov (95)	Group1[b) Autism: Ctrl]	Group2[b) Autism: Ctrl]	Group3[b) Autism: Ctrl]	References
Actin, cytoplasmic 2 (−)[a)]	P63261	ACTG1	24	46	−1.91	−4.15	−3.34	[20,21]
Alpha-1-antitrypsin (+)[c)]	P01009	SERPINA1	37	50	2.07	2.94	4.27	[42,43]
Alpha-actinin-1 (−)	P12814	ACTN1	28	31	−3.91	−4.89	−4.21	[56,57]
Alpha-enolase (−)	P06733	ENO1	6	19	−1.02	−1.87	−2.34	[58]
Angiotensinogen (+) [c)]	P01019	AGT	21	31	3.31	5.71	4.77	[59]
Apolipoprotein E (+) [c)]	P02649	APOE	136	68	1.99	1.42	3.61	[47]
Beta-parvin (−)	Q9HBI1	PARVB	7	17	−2.79	−5.05	−3.45	[23]
Calmodulin (−)	P62158	CALM1	5	50	−3.27	−4.41	−4.27	[29,60]
Calreticulin (−)	P27797	CALR	5	16	−2.18	−2.22	−1.86	[30,61]
Complement C3 (+) [c)]	P01024	C3	156	49	2.91	3.00	2.96	[40]
Complement C5 (+)	P01031	C5	14	10	2.06	2.39	1.93	No[d)]
EH domain-containing -containing protein 3 (−)	Q9NZN3	EHD3	10	19	−2.76	−4.03	−4.52	[52]
Fermitin family homolog 3 (−)	Q86UX7	FERMT3	19	37	−2.43	−3.43	−4.09	No[d)]
Fibronectin (+) [c)]	P02751	FN1	65	30	1.26	2.07	3.76	[25,61,62]
Fibulin-1 (+)	P23142	FBLN1	20	15	1.18	1.40	2.41	[31]
Insulin-like growth factor -binding protein complex acid labile subunit (+)	P35858	IGFALS	9	17	0.82	3.32	1.97	[57]
Integrin alpha-IIb (−)	P08514	ITGA2B	6	5.6	−2.03	−2.34	−3.23	[26]
Kallistatin (+) [c)]	P29622	SERPINA4	7	19	1.67	2.72	1.00	[59]
Microtubule-associated protein RP/EB family member 2 (−)	Q15555	MAPRE2	3	10	−2.80	−4.56	−3.67	[53]
Talin-1 (−)	Q9Y490	TLN1	90	32	−2.82	−4.36	−4.90	[39]
Thrombospondin-1 (−)	P07996	THBS1	31	22	−1.79	−1.65	−3.65	[28,60]
Transitional endoplasmic reticulum ATPase (−)	P55072	VCP	21	31	−1.05	−3.76	−3.26	[54,63]
Vinculin (−)	P18206	VCL	22	23	−2.18	−3.16	−4.09	[22]
Vitronectin (+)	P04004	VTN	141	53	1.65	3.11	2.41	[27]

a) (+), protein increased in abundant; (−), protein decreased in abundant.
b) Fold change ($\log_2$ ratio), $p < 0.05$ versus the control.
c) The protein has been identified as differentially expressed protein in the blood of autistic patients in previous reports or patents.
d) No, not been reported.

p-value based on the full list of identified proteins (Supporting Information, Table 2). Among 24 differentially expressed proteins, nine proteins including ACTN1, AGT, C3, C5, FN1, SERPINA1, THBS1, TLN1, and VTN were found to be significant in the three comparisons, and nine proteins include ACTG1, APOE, FBLN1, FERMT3, IGFALS, ITGA2B, PARVB, VCL, and VCP were significant in the two comparisons (Supporting Information, Table 2).

3.3. Bioinformatics Analysis of the Differentially Expressed Proteins

The differentially expressed proteins were analyzed by different databases. In DAVID analysis (**Figure 2A–C**), we used the list of all detected proteins as the baseline, the results showed that the majority of the differentially expressed proteins are associated with focal adhesion, regulation of cell migration and cell motility, cell adhesion, cell junction, blood coagulation, hemostasis, response to wounding, platelet activation and degranulation, Rap1 signaling pathway, and MAP2K and MAPK activation, suggesting that these proteins and the biochemical processes link to them might play a critical role in the pathogenesis of autistic disorder. In addition, there are 17 pathways that were enriched (Figure 2B), among them, the *p*-value of four pathways, that is, MAP2K and MAPK activation, syndecan interactions, focal adhesion, and Rap1 signaling pathway was less than 0.05 when using the list of all detected proteins as the baseline; however, when we used *Homo sapiens* as background, except for PI3K-Akt signaling pathway, the *p*-value of all the other pathways was less than 0.05 (data not shown). The results implied that MAP2K and MAPK activation,

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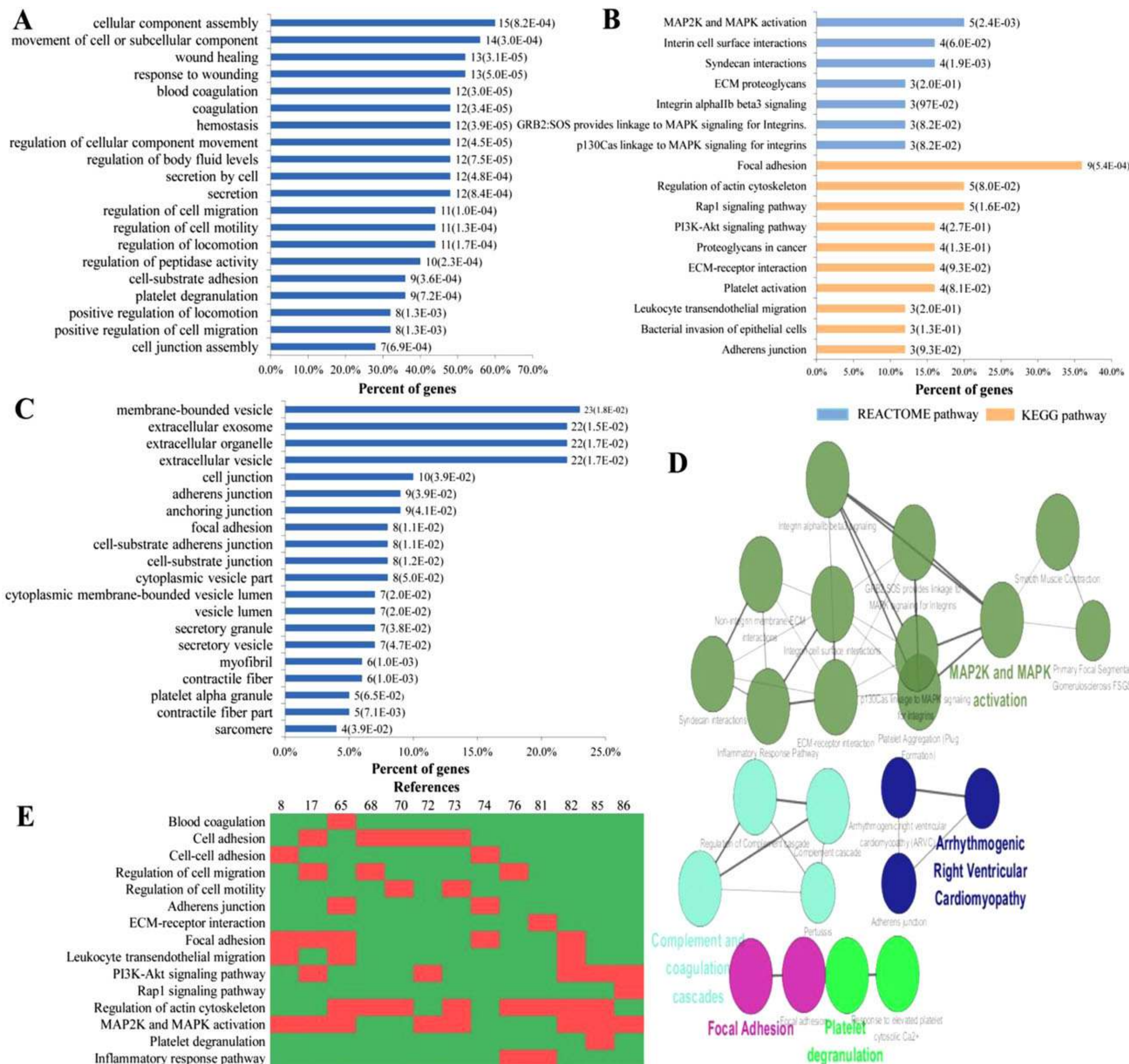

Figure 2. Bioinformatics analysis of differentially expressed proteins in the plasma of autistic and healthy subjects. (A) The top 20 ranking of biological process based on gene ontology (GO). (B) The signal transduction pathways are associated with these proteins. (C) The top 20 ranking of cellular components based on GO. (A–C) Number of proteins associated with each category and *p*-value for gene-enrichment analysis are shown on the right of each term bar. (D) The differentially expressed proteins were mapped to the GO categories (biological processes), KEGG pathway, REACTOME pathway, and Wiki pathway by using ClueGO cytoscape plugin. (E) The literatures have more than two pathways overlapping with the present study.

syndecan interactions, focal adhesion, and Rap1 signaling pathway may be more important in the pathogenesis of autism.

We used the Cytoscape software with ClueGO plug-in to further investigate the functional interaction networks of differentially expressed proteins. The results are consistent with DAVID analysis (Figure 2D), these proteins were mainly involved in cell–substrate adhesion, focal adhesion, extracellular matrix (ECM)–receptor interaction, platelet degranulation, etc. Besides, the inflammatory response, complement and coagulation cascades, MAP2K and MAPK activation, integrin alphaIIb beta3 signaling, and platelet aggregation pathways were also enriched. The biological processes and pathways related to these proteins are listed in **Table 2**. Interestingly, except for the pathway of response to elevated platelet cytosolic Ca^{2+}, all of these signal transduction pathways have been reported to be associated with autism in the previous studies. It is worth noting that 13 studies have shown more than two pathways overlapping with the present study and these references and pathways are presented in Figure 2E. For example, it is interesting that those three pathways, that is, focal adhesion, cell adhesion molecules, and leukocyte transendothelial migration, were related to Han Chinese cohort in a recent study.[8]

Subsequently, PPI networks were generated and are shown in **Figure 3**. Twenty-five differentially expressed proteins (24

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Table 2. The differentially expressed proteins associated with biological processes and signal transduction pathways.

Gene ontology/pathways	Pathways	Count	Genes	References
Biological processes	Blood coagulation	15	ACTG1,ACTN1,APOE,C3,CALM1,EHD3,FBLN1,FERMT3, FN1,ITGA2B,SERPINA1,THBS1,TLN1,VCL,VTN	[64,65]
	Cell adhesion	14	ACTG1,ACTN1,ACT,CALR,FERMT3,FN1,FBLN1,ITGA2B, PARVB,TLN1,THBS1,VCL,IGFALS	[3,17,66–73]
	Cell–cell adhesion	6	ACTN1,ACTG1,FERMT3,ITGA2B,TLN1,VCL	[8,74]
	Cell–substrate adhesion	10	ACTN1,AGT,CALR,FERMT3,FN1,FBLN1,ITGA2B,THBS1, VCL,VTN	[75]
	Regulation of cell migration	11	AGT,APOE,CALR,C5,FERMT3,FN1,FBLN1,ITGA2B,THBS1, VCL,VTN	[17,68,76–78]
	Regulation of cell motility	11	ACT,APOE,CALR,C5,FERMT3,FN1,ITGA2B,THBS1,VCL, VTN,FBLN1	[70,73]
KEGG pathway	Adherens junction	3	ACTG1, ACTN1, VCL	[65,74]
	Bacterial invasion of epithelial cells	3	ACTG1,FN1,VCL	[79]
	ECM–receptor interaction	4	FN1,ITGA2B,THBS1,VTN	[80,81]
	Focal adhesion[a)]	9	ACTG1,ACTN1,FN1,ITGA2B,PARVB,THBS1,TLN1,VCL,VTN	[8,17,65,66,74,82]
	Leukocyte transendothelial migration	3	ACTG1,ACTN1,VCL	[8,65,82,83]
	PI3K-Akt signaling pathway	4	FN1,VTN,ITGA2B,THBS1	[17,72,82,84–87]
	Platelet activation	4	ACTG1,FERMT3,ITGA2B,TLN1	[33,34]
	Proteoglycans in cancer	4	ACTG1,FN1,VTN	[88]
	Rap1 signaling pathway	5	ACTG1,CALM1,ITGA2B,THBS1,TLN1	[86]
	Regulation of actin cytoskeleton	5	ACTG1,ACTN1,FN1,ITGA2B,VCL	[65,68,70,73,76,81,82,85]
REACTOME	Complement cascade[a)]	3	C3, C5, VTN	[25,89,90]
	Integrin alphaIIb beta3 signaling	3	FN1, ITGA2B, TLN1	[38]
	MAP2K and MAPK activation	4	FN1, ITGA2B, TLN1, VCL	[8,17,65,72,73,82,83,85,86,91]
	Platelet aggregation	3	FN1, ITGA2B, TLN1	[34,38]
	Platelet degranulation	10	ACTN1, CALM1, FERMT3, FN1, ITGA2B, SERPINA1, SERPINA4, THBS1, TLN1, VCL	[85]
	Response to elevated platelet cytosolic Ca^{2+}	10	ACTN1, CALM1, FERMT3, FN1, ITGA2B, SERPINA1, SERPINA4, THBS1, TLN1, VCL	No[b)]
Wiki Pathways	Inflammatory response pathway	3	FN1, THBS1, VTN	[44,57,76,81,89,92–94]

a) These pathways were also enriched in the Wiki pathways.
b) No, not been reported.

differentially expressed proteins as mentioned above plus GC) were found interacting with each other in a protein interaction network (Figure 3A). With these proteins as candidates and autism-related genes as target genes, the other PPI networks were generated (Figure 3B). All of 25 proteins and 5 autism-related genes (CDH9, CDH10, PARD3B, PPP2R5C, and TAF1C) were enriched in the interaction networks. Likewise, other PPI networks were generated by using 25 differentially expressed proteins as candidates and 44 combined genes (ASD, attention deficit hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia; NHGRI GWAS; Figure 3C) as target genes. As shown in Figure 3C, all of candidate genes and 22 target genes (ANK3, ANKS1B, CACNA1C, CACNB2, DCP1B, DPYD, ENO1, GRIN2A, HDAC4, KIF5C, MAP4K4, MMP16, NT5C2, NTRK3, PPP2R2B, RERE, SMARCA2, SYNE1, TCF4, ZFPM2, ZMIZ1, and ZNF804A) among the 44 combined genes were enriched in an interaction networks. Of which ENO1 was found common gene between candidate genes and target genes. Interestingly, comparing the two PPI networks of different target genes (Figure 3B and C), more proteins were interacted in the networks of autism-related genes as target genes, suggesting that these differentially expressed proteins were tightly

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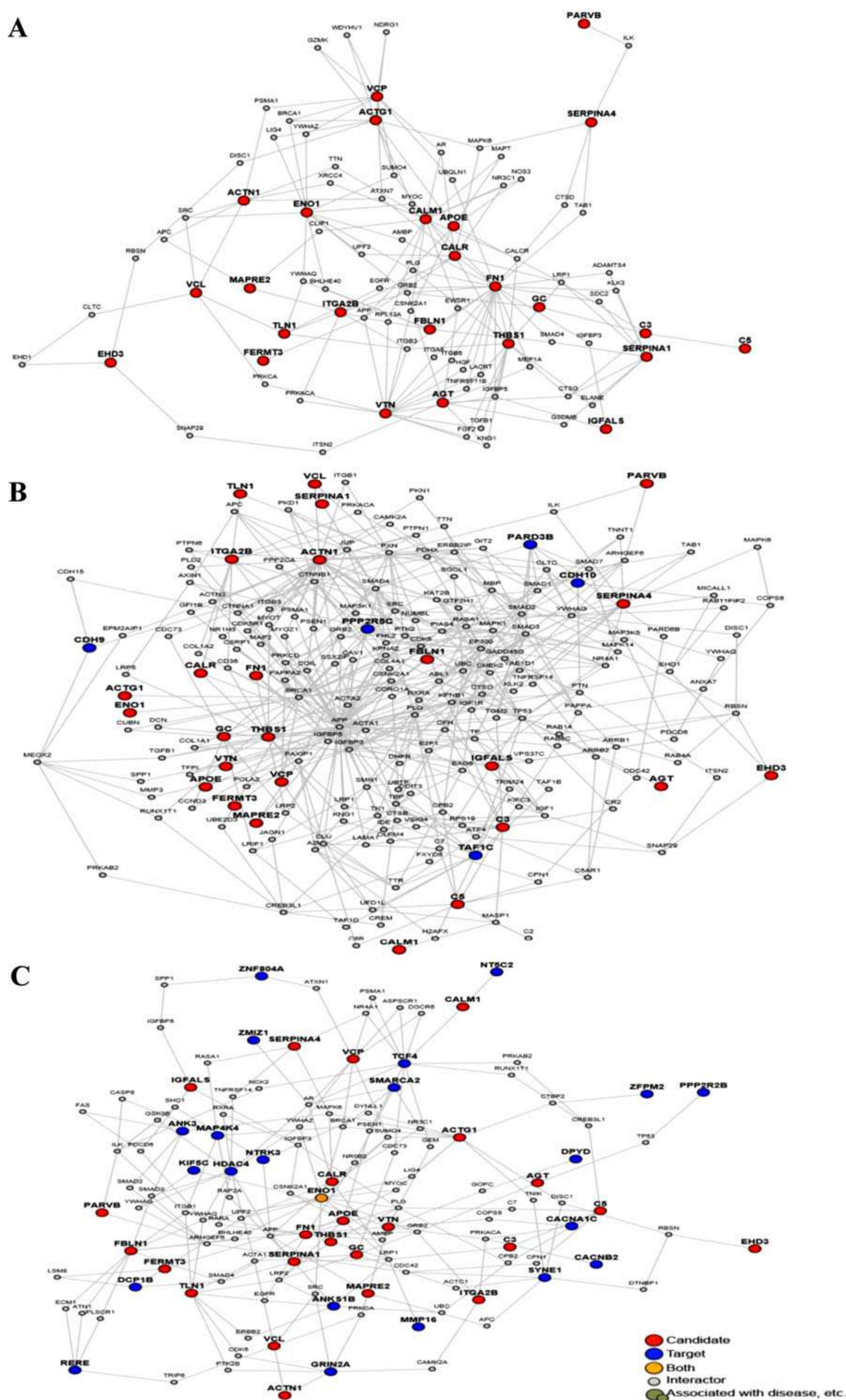

Figure 3. Protein–protein interaction networks of differentially expressed plasma proteins in autistic children and healthy subjects. The networks analysis was carried out by using LENS. (A) Network corresponding only to differentially expressed proteins (shown in red) shows the immediate interactions and paths between them. (B) Network generated with the differentially expressed proteins as candidate genes (shown in red) and eight autism genes (NHGRI GWAS) include CDH9, CDH10, MACROD2, PARD3B, PPP2R5C, SEMA5A, TAF1C, and TAS2R1 as target genes (shown in blue). (C) Network generated with the differentially expressed proteins as candidate genes (shown in red) and combined genes (ASD, attention deficit hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia, NHGRI GWAS) as target genes (shown in blue), including ANK3, ANKS1B, ANO5, AS3MT, BANK1, CACNA1C, CACNB2, CCDC68, CNNM2, CSMD1, DCP1B, DPYD, ELTD1, ENO1, FAM155A, GRIK1, GRIN2A, HDAC4, IFI44, IFI44L, IL1R2, ITIH3, KIF5C, LYPD6B, MAP4K4, MGC16025, MIR137, MMP16, NT5C2, NTRK3, PCGEM1, PPP2R2B, RERE, SEMA3A, SLC17A6, SLC35F2, SLC45A1, SMARCA2, STT3A, SYNE1, TCF4, ZFPM2, ZMIZ1, and ZNF804A. NHGRI, National Human Genome Research Institute; GWAS, genome-wide association studies.

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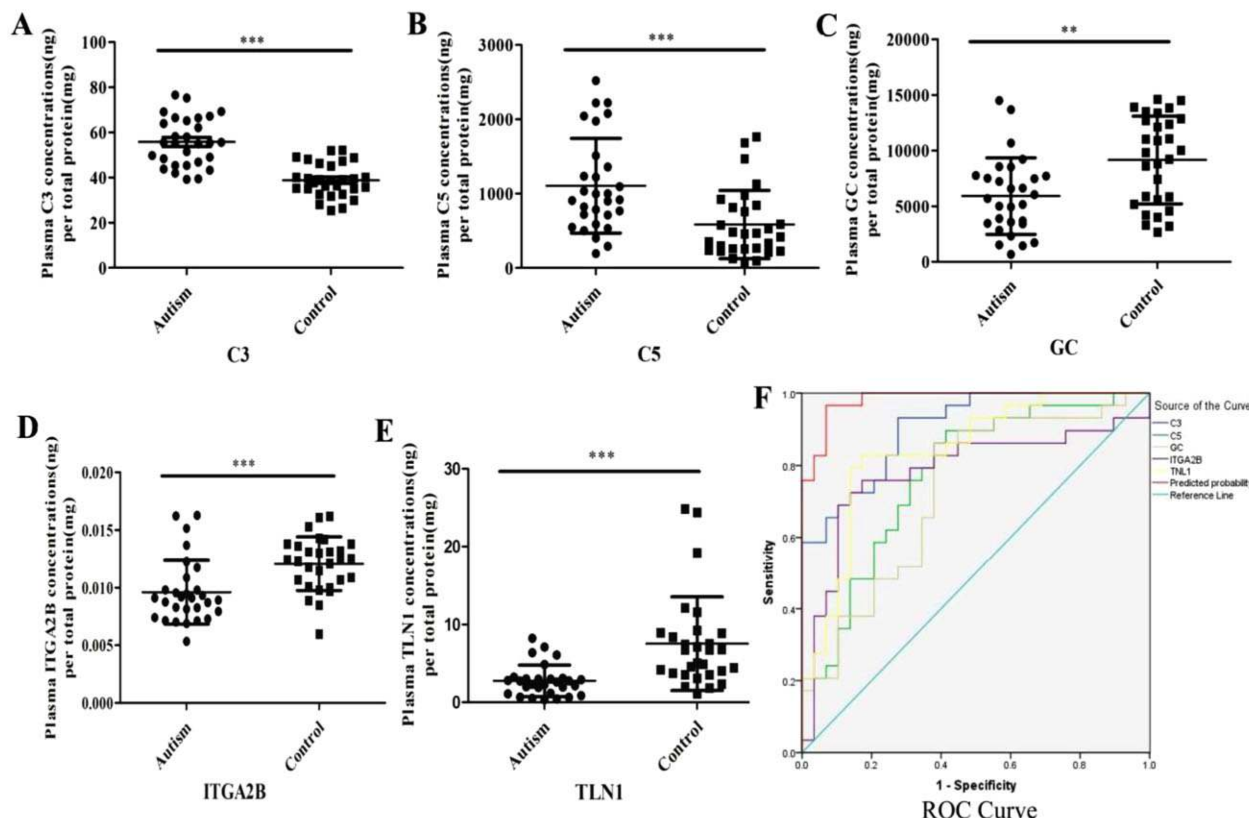

Figure 4. Validation of differentially expressed proteins in the plasma of children with autism by ELISA. The graphical results depict the mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$. (A) Complement C3 (C3). (B) Complement C5 (C5). (C) Integrin alpha-IIb (ITGA2B). (D) Talin-1 (TLN1). (E) Vitamin D binding protein (GC). (F) ROC curve analysis. ROC curves analysis of C3, C5, GC, ITGA2B, TLN1 and combine C3, C5, ITGA2B, TLN1 to discriminate autistic patients from healthy controls.

associated with autism-related genes than the other psychiatric disorders. The network statistics associated to the LENS analysis is presented in Supporting Information, Table 3. Three values include minimum shortest path length, average shortest path length, and the count of disconnected nodes is used to describe the network connectivity. The results showed that these three values were higher in random genes than that in candidate genes when only candidate genes were given, whereas in candidate to target were lower than that in candidate to random when both candidate and target genes were given. The results supported that these networks are well enriched.

3.4. ELISA Validation

We selected five proteins involved in different signal transduction pathways and we could obtain their commercial ELISA kits, including C3, C5, GC, ITGA2B, and TLN1 to be validated by ELISA assay. Among them, C3, C5, and TLN1 were significantly changed in the three comparisons through FDR correction of p-value. In addition, the fold changes of GC were higher and variation trends were consist in three groups, and exhibited differential expression in two groups ($p < 0.05$) was also verified by ELISA analysis. As shown in **Figure 4**, consistent with the iTRAQ analysis, the plasma levels of C3 and C5 were increased in autistic children compared with controls (Figure 4A, B, $p < 0.05$), whereas the plasma levels of GC, ITGA2B, and TLN1 in autistic patients were significantly lower than those in healthy controls (Figure 4C–E, $p< 0.05$). These data validate the iTRAQ analysis results and suggest that the plasma levels of C3, C5, GC, ITGA2B, and TLN1 may serve as potential biomarker for the diagnosis of autism. The ELISA results for these five proteins were used for ROC analysis, AUC values of C3, C5, ITGA2B, GC, and TLN1 were 0.904 (95% CI, 0.831–0.977, $p < 0.000$), 0.773 (95% CI, 0.651–0.894, $p < 0.000$), 0.782 (95% CI, 0.653–0.912, $p < 0.000$), 0.728 (95% CI, 0.596–0.860, $p < 0.003$), and 0.839 (95% CI, 0.735–0.944, $p < 0.000$), respectively. Combining five proteins, that is, C3, C5, GC, ITGA2B, and TLN1 were used to generate the ROC curves. Figure 4F shows that AUC value of combinations of these five proteins is 0.982 (95% CI, 0.957–1.000, $p < 0.000$), resulting in a higher AUC value than that of a single protein.

4. Discussion

The present proteomics study revealed 24 differentially expressed plasma proteins in the autistic subjects compared to healthy controls. Among these, 13 proteins are found involved in focal adhesions, regulation of cytoskeleton, cell motility and migration, cell adhesion, adherens junction, and ECM–receptor interaction, which include ACTG1, ACTN1, C5, CALR, FERMT3, FN1,

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FBLN1, ITGA2B, PARVB, TLN1, THBS1, VCL, and IGFALS. These processes have been reported to be associated with ASD in the previous studies and our results were similar to these studies. For example, expression of FAK (focal adhesion kinase) was diminished in autistic lymphoblasts and overexpression of FAK in these cells restored the adhesion and migration defects.[17] Besides ASDs, change in cytoskeleton proteins expression has been reported in other psychiatric disorders.[18] In addition, recent genetic studies demonstrate that alterations in synaptic genes including those encoding cell adhesion molecules and their interaction partners play important roles in the pathogenesis of ASD.[19] Migration defects have also been proposed as one of the major pathways of autism.[17]

Out of these 13 proteins, ACTG1 has been identified as autism-associated gene,[20,21] and VCL protein is the partner of SHANK3 (a susceptibility gene for ASD).[22] PARVB is involved in integrin-mediated cell adhesion, regulation of cell spreading, and cell motility. Deletion of this gene leads to macrocephaly and overgrowth and associated with a markedly enlarged brain.[23] Likewise, there are three proteins, that is, FN1, ITGA2B, and TLN1 involved in integrin alphaIIb beta3 signaling. Integrin-mediated signaling events in neurons regulate glutamate receptor activity and downstream control of the neuronal cytoskeleton.[24] FN1 is an extracellular matrix protein involved in cell adhesion and migration.[25] Consistent with a previous study,[25] the expression levels of FN1 were upregulated in autistic children in this study. ITGA2B has been proposed as a candidate gene for autism, which is related to cell adhesion and cell-surface-mediated signaling and links the extracellular matrix to the intracellular cytoskeleton via integration with ITGB3 forms αIIbß3.[26] Its expression levels were found to be increased in the ASD brain and peripheral tissues.[26] On the contrary, here, the lower levels of ITGA2B were detected in autism plasma, and thus need to be further clarified. Another protein VTN has been demonstrated to interact with the cell adhesion molecule, a close homolog of L1 (CHL1). CHL1 plays important roles in the development and the adult nervous system and links to mental retardation including ASD.[27] Additionally, the process of synapse formation, or synaptogenesis, is tightly regulated to ensure correct connections between neurons. Most of susceptible or causative genes of ASD are related to synaptogenesis.[28] In this study, three proteins (TSP-1, CALM1, and CALR) associated with synaptogenesis are observed to be decreased, whereas one protein (FBLN1) is found increased in the plasma of autistic subjects. TSP-1 protein encoded by THBS1 gene is an important astrocyte-secreted protein being involved in the regulation of spine development and synaptogenesis. Particularly, it has been proposed as a novel susceptible gene for autism in the Chinese Han population.[28] CALM1 plays a critical role in regulating voltage-independent calcium-activated action potentials at the neuronal synapse, being proposed as a candidate gene for the ASD.[29] CALR and FBLN1 were found to be candidate chaperone proteins interacting with autism-associated NLGN3 (Neurolign 3) gene.[30,31]

Moreover, ten proteins, that is, ACTN1, CALM1, FERMT3, FN1, ITGA2B, SERPINA1, SERPINA4, THBS1, TLN1, and VCL, are associated with platelet function and response to elevated platelet cytosolic Ca^{2+}. Interestingly, the previous studies indicated that the platelets and neurosecretory cells have common gene expression profiles and share several biological features. Changes in platelet functions were often detected in monogenetic and complex neurological diseases including autism.[32] For example, platelet activity has been detected to be decreased in infantile autism.[33] A recent study showed that increased platelet counts, decreased platelet ATP dense granule secretion, and increased serotonin plasma levels not only in ASD patients, but also in their first-degree relatives.[34] Serotonin could induce increased platelet cytosolic calcium concentration in depressed[35] and bipolar affective disorder.[36] Our results showed that 10 proteins are found associated with response to elevated platelet cytosolic Ca^{2+}. Thus, it can be hypothesized that platelet cytosolic calcium concentration may be increased in the autistic children and induced by serotonin. Indeed, affected genes include FOXG1, AQP7, GNAS, and ADCYAP1 (PACAP) with modifications of the platelet aggregation response and/or variations in dense or agranule number or release have been reported.[32] Some signal transduction pathways associated with serotonin metabolism such as platelet activation, degranulation, and aggregation have been reviewed, and they may be involved in autism during early brain development.[37] In addition, integrin αIIbβ3 interacts with antidepressant-sensitive serotonin (5-HT) transporter SERT may have broad implications for hyperserotonemia and autism.[38] Besides, FERMT3, an integrin-activating protein, implicated in platelet function, is being reported for the first time to be decreased in the plasma of autistic patients. A recent study suggested that TNL1 may be involved in remodeling of the actin cytoskeleton of platelets.[39] Therefore, the results agree with the fact that platelet dysfunction may play a critical role in the pathogenesis of autism, and these proteins identified in this study are associated with platelet function might contribute to autism pathophysiology.

Four proteins, that is, C3, C5, SERPINA1, and VTN, are found involved in complement and coagulation cascades. Similarly, compared to healthy controls, several upregulated complement proteins have been reported in the serum of ASD.[25,40] Coinciding with our results, increase in three peptides that correspond to C3 complement protein fragments were identified in the blood of children with ASD in a previous study.[40] For the first time, C5 is found to be increased in the plasma of autistic children in this study. However, a very recent study reported that C3 mRNA levels were decreased in the middle frontal gyrus of ASD subjects compared to controls.[41] Complement proteins are produced mainly by the liver.[41] As the blood–brain barrier (BBB) exists, the brain produces complement proteins locally. Thus, the complement system in the periphery of subjects with ASD may be activated.[41] Low levels of SERPINA1 have been observed in some children with autism,[42,43] which is inconsistent with our finding and need further confirmation. Activation of complement pathway can ultimately lead to the release of inflammatory mediators. Increased levels of inflammatory markers have been reported in individuals with autism and immune dysfunction being a potential mechanism for ASD.[44] Postmortem brain studies on the possible effects on inflammation in ASD have also been conducted previously.[45] In the present study, three proteins, that is, FN1, THBS1, and VTN, were found associated with inflammatory reaction. Hence, our results support the view that the complement system and inflammatory reactions might be involved in the pathophysiology of autism.

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Apolipoproteins (apos) are the protein component of lipoproteins involved in the transport of lipids, cholesterol, and vitamin E in the circulation. The changes in apos have already been reported in the serum of ASD.[40,46] APOE is a significant factor in the neurodegenerative diseases. Here, it was found to be increased in the plasma of autistic patients, this is similar with a previous study.[47] However, another previous study found no evidence for linkage or association of APOE to autism.[48] Thus, the role of APOE in autism pathogenesis needs to be studied further. Another protein, vitamin D binding protein (GC), was found to be significantly downregulated in the plasma of autistic children. Vitamin D deficiency, either in pregnancy or during postnatal development, is an apparent risk factor for autism.[49] GC is suggested to help in binding vitamin D in the central nervous system. A previous report showed that its levels were increased in newborns serum, developing ASD later in life due to low vitamin D level.[50] This inconsistency with our results may be due to the age differences, being 2–6 years old in the present study, but it is newborns in the literature.[50]

Moreover, the other proteins such as EHD3, MAPRE2, and VCP have also been found related to autism.[51–54] EHD3 is a family member of the human EH domain containing proteins and plays a role as a linker between the premature endosome and the endocytic recycling compartment. It has been associated with major depressive disorder in the Chinese population[51] and linked to autism in a recent study.[52] MAPRE2 is a microtubule-associated protein that is possibly involved in the development of neuronal processes and associated with autism.[53] VCP proteins are ubiquitously expressed in various tissues. It influences the efficiency of protein synthesis to control dendritic spine formation in neurons,[55] and mutations in the VCP gene results in multisystem disorders including ASD.[54] Our results agree with these three proteins may contribute to the pathophysiology of autism. Furthermore, five proteins, that is, C3, C5, GC, ITGA2B, and TLN1 were successfully validated by ELISA analysis. The results were consistent with the LC/MS quantification results. By ROC curve analysis, the results showed that combining five proteins (C3, C5, ITGA2B, GC and TLN1) lead to a higher AUC value, suggesting that they may serve as a biomarker panel to aid in the diagnosis of autism.

In conclusion, this is the first study to investigate the behavior of plasma proteins in autistic children compared to healthy controls by using iTRAQ-based proteomic approach. Differentially expressed proteins and the signal transduction pathways related to autism are identified here. Most of them have previously been linked to the pathophysiology of ASD. The proteins may serve as candidate markers and might be used for diagnosis of this disease in the future. These findings support current theories regarding ASD onset, and highlight the roles of several networks in the autistic pathogenesis, including focal adhesions, acting cytoskeleton, cell adhesion, cell motility and migration, synaptogenesis, complement system, and platelet function, suggesting that autism is a multifactorial disorder with complicated pathophysiological changes. However, this study is a preliminary investigation regarding biomarker discovery for ASD and give a clue for further research. For more authentic results, further study is required on large scale with large population size. Moreover, further validation of above-mentioned differentially expressed proteins will make the results more valid and practical. Age limitations should also be addressed in future studies for better understanding of the results. The other potential limitations and confounders include the patient heterogeneity (e.g., disease severity), these findings correlate with disease-related behaviors, the experimental design, and lack of control of a number of variables (e.g., non-ASD control group with developmental delays and/or other disorders of childhood), etc. In addition, it will be interesting to further investigate the same changes of these proteins whether or not occur in the brain tissue or peripheral blood cell/platelet of autistic children.

Abbreviations

95% CI, 95% confidence interval; ASD, autism spectrum disorder; AUC, area under the curve; EF, error factor; iTRAQ, isobaric tags for relative and absolute quantitation; PPI, protein–protein interaction; ROC, receiver operating characteristic

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declared no conflict of interest.

Keywords

autism, biomarkers, iTRAQ, plasma, proteomics

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[1] V. Mandic-Maravic, M. Pejovic-Milovancevic, M. Mitkovic-Voncina, M. Kostic, O. Aleksic-Hil, J. Radosavljev-Kircanski, T. Mincic, D. Lecic-Tosevski, *Sci. Rep.* **2015**, *5*, 10418.
[2] M. Fakhoury, *Int. J. Dev. Neurosci.* **2015**, *43*, 70.
[3] C. Betancur, T. Sakurai, J. D. Buxbaum, *Trends Neurosci.* **2009**, *32*, 402.
[4] S. Tordjman, E. Somogyi, N. Coulon, S. Kermarrec, D. Cohen, G. Bronsard, O. Bonnot, C. Weismann-Arcache, M. Botbol, B. Lauth, V. Ginchat, P. Roubertoux, M. Barburoth, V. Kovess, M. M. Geoffray, J. Xavier, *Front. Psychiatry* **2014**, *5*, 53.
[5] E. Fernell, M. A. Eriksson, C. Gillberg, *Clin. Epidemiol.* **2013**, *5*, 33.
[6] A. M. Daniels, D. S. Mandell, *Autism* **2014**, *18*, 583.
[7] E. Fernell, C. Gillberg, *Res. Dev. Dis.* **2010**, *31*, 680.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[8] E. Skafidas, R. Testa, D. Zantomio, G. Chana, I. P. Everall, C. Pantelis, *Mol. Psychiatry* **2014**, *19*, 504.
[9] J. A. S. Vorstman, J. R. Parr, D. Moreno-De-Luca, R. J. L. Anney, J. I. Nurnberger Jr., J. F. Hallmayer, *Nat. Rev. Genet.* **2017**, *18*, 362.
[10] M. Stamou, K. M. Streifel, P. E. Goines, P. J. Lein, *Neurotoxicol. Teratol.* **2013**, *36*, 3.
[11] N. J. Minshew, T. A. Keller, *Curr. Opin. Neurol.* **2010**, *23*, 124.
[12] L. Shen, L. Liao, C. Chen, Y. Guo, D. Song, Y. Wang, Y. Chen, K. Zhang, M. Ying, S. Li, Q. Liu, J. Ni, *J. Alzheimer's Dis.* **2017**, *56*, 361.
[13] D. Zhao, L. Shen, Y. Wei, J. Xie, S. Chen, Y. Liang, Y. Chen, H. Wu, *Proteomics Clin. Appl.* **2017**, *11*, 1600152.
[14] I. Ruppen, L. Grau, E. Orenes-Piñero, K. Ashman, M. Gil, F. Algaba, J. Bellmunt, M. Sánchez-Carbayo, *Mol. Cell. Proteomics* **2010**, *9*, 2276.
[15] G. Bindea, B. Mlecnik, H. Hackl, P. Charoentong, M. Tosolini, A. Kirilovsky, W. H. Fridman, F. Pagès, Z. Trajanoski, J. Galon, *Bioinformatics* **2009**, *25*, 1091.
[16] A. Handen, M. K. Ganapathiraju, *BMC Med. Genomics* **2015**, *8*(Suppl. 4), S2.
[17] H. Wei, M. Malik, A. M. Sheikh, G. Merz, B., W.T., X. Li, *Am. J. Pathol.* **2011**, *179*, 66.
[18] J. V. Coumans, S. K. Palanisamy, J. McFarlane, P. D. Moens, *Int. J. Mol. Sci.* **2016**, *17*, 518.
[19] H. Ye, J. Liu, J. Y. Wu, *Neurosignals* **2010**, *18*, 62.
[20] B. E. Eisinger, M. C. Saul, T. M. Driessen, S. C. Gammie, *BMC Neurosc*. **2013**, *14*, 147.
[21] E. L. Casanova, J. L. Sharp, H. Chakraborty, N. S. Sumi, M. F. Casanova, *Mol. Autism* **2016**, *7*, 18.
[22] C. M. Durand, J. Perroy, F. Loll, D. Perrais, L. Fagni, T. Bourgeron, M. Montcouquiol, N. Sans, *Mol. Psychiatry* **2012**, *17*, 71.
[23] V. Disciglio, C. Lo Rizzo, M. A. Mencarelli, M. Mucciolo, A. Marozza, C. Di Marco, A. Massarelli, V. Canocchi, M. Baldassarri, E. Ndoni, E. Frullanti, S. Amabile, B. M. Anderlid, K. Metcalfe, C. Le Caignec, A. David, A. Fryer, O. Boute, A. Joris, D. Greco, V. Pecile, R. Battini, A. Novelli, M. Fichera, C. Romano, F. Mari, A. Renieri, *Am. J. Med. Genet. A* **2014**, *164A*, 1666.
[24] M. E. Kerrisk, L. A. Cingolani, A. J. Koleske, *Prog. Brain Res.* **2014**, *214*, 101.
[25] N. Momeni, J. Bergquist, L. Brudin, F. Behnia, B. Sivberg, M. T. Joghataei, B. L. Persson, *Transl. Psychiatry*, **2012**, *2*, e91.
[26] Y. Kuwano, Y. Kamio, T. Kawai, S. Katsuura, N. Inada, A. Takaki, K. Rokutan, *PLoS One* **2011**, *6*, e24723.
[27] J. Katic, G. Loers, R. Kleene, N. Karl, C. Schmidt, F. Buck, J. W. Zmijewski, I. Jakovcevski, K. T. Preissner, M. Schachner, *J. Neurosci.* **2014**, *34*, 14606.
[28] L. Lu, H. Guo, Y. Peng, G. Xun, Y. Liu, Z. Xiong, D. Tian, Y. Liu, W. Li, X. Xu, J. Zhao, Z. Hu, K. Xia, *Psychiatric Genet.* **2014**, *24*, 235.
[29] D. Hadley, Z. L. Wu, C. Kao, A. Kini, A. Mohamed-Hadley, K. Thomas, L. Vazquez, H. Qiu, F. Mentch, R. Pellegrino, C. Kim, J. Connolly, A. G. P. Consortium, J. Glessner, H. Hakonarson, *Nat. Commun.* **2014**, *5*, 4074.
[30] A. De Jaco, D. Comoletti, C. C. King, P. Taylor, *Chem. Biol. Interact.* **2008**, *175*, 349.
[31] C. Shen, L. Huo, X. Zhao, P. Wang, N. Zhong, *J. Mol. Neurosci.* **2015**, *56*, 89.
[32] C. Goubau, G. M. Buyse, C. Van Geet, K. Freson, *Dev. Med. Child Neurol.* **2014**, *56*, 724.
[33] S. Safai-Kutti, J. Kutti, C. Gillberg, *Acta Paediatr.* **1985**, *74*, 799.
[34] N. Bijl, C. Thys, C. Wittevrongel, W. De la Marche, K. Devriendt, H. Peeters, C. Van Geet, K. Freson, *Mol. Autism* **2015**, *6*, 57.
[35] L. M. Konopka, R. Cooper, J. W. Crayton, *Biol. Psychiatry* **1996**, *8*, 708.
[36] L. Wöckel, F. D. Zepf, S. Koch, A.-E. Meyer-Keitel, M. H. Schmidt, *J. Neural Transm.* **2008**, *116*, 89.
[37] C. Yang, H. Tan, Y. Du, *Neuroscience* **2014**, *267*, 1.
[38] A. M. Carneiro, E. H. Cook, D. L. Murphy, R. D. Blakely, *J. Clin. Invest.* **2008**, *118*: 1544.
[39] K. Nuytens, K. Tuand, M. Di Michele, K. Boonen, E. Waelkens, K. Freson, J. W. Creemers, *Mol. Autism* **2013**, *4*, 43.
[40] B. A. Corbett, A. B. Kantor, H. Schulman, W. L. Walker, L. Lit, P. Ashwood, D. M. Rocke, F. R. Sharp, *Mol. Psychiatry* **2007**, *12*, 292.
[41] K. Fagan, A. Crider, A. O. Ahmed, A. Pillai, *Mol. Neuropsychiatry* **2017**, *3*, 19.
[42] A. J. Russo, L. Neville, C. Wroge, *Biomark. Insights* **2009**, *4*, 45.
[43] A. J. Russo, A. Krigsman, B. Jepson, A. Wakefield, *Genomics Insights* **2009**, *2*, 1.
[44] N. Xu, X. Li, Y. Zhong, *Mediators Inflamm*. **2015**, *2015*, 531518.
[45] J. I. Rodriguez, J. K. Kern, *Neuron Glia Biol.* **2011**, *7*, 205.
[46] N. Wetie, G. Armand, K. Wormwood, J. Thome, E. Dudley, R. Taurines, M. Gerlach, A. G. Woods, C. C. Darie, *Electrophoresis* **2014**, *35*, 2046.
[47] H. Steeb, J. M. Ramsey, P. C. Guest, P. Stocki, J. D. Cooper, H. Rahmoune, E. Ingudomnukul, B. Auyeung, L. Ruta, S. Baron-Cohen, S. Bahn *Mol. Autism* **2014**, *5*, 4.
[48] K. L. Raiford, Y. Shao, I. C. Allen, E. R. Martin, M. M. Menold, H. H. Wright, R. K. Abramson, G. Worley, G. R. DeLong, J. M. Vance, M. L. Cuccaro, J. R. Gilbert, M. A. Pericak-Vance *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **2004**, *125B*, 57.
[49] E. Kocovska, E. Fernell, E. Billstedt, H. Minnis, C. Gillberg *Res. Dev. Disabil.* **2012**, *33*, 1541.
[50] M. Hornig, *Vitamin D Binding Protein as a Biomarker for Autism Enables Early Detection and Treatment*. Columbia Technology Ventures, New York.
[51] C. Shi, K. Zhang, X. Wang, Y. Shen, Q. Xu *Am. J. Med. Genet. B* **2012**, *159*, 336.
[52] B. Gudenas, L. Wang, *Graduate Res. Discovery Symp*, TigerPrints, Clemson, SC **2015**.
[53] M. G. Nielsen Chromosomal abnormalities and putative susceptibility genes in autism spectrum disorders. PhD Thesis, Københavns Universitet **2007**.
[54] I. Iossifov, M. Ronemus, D. Levy, Z. Wang, I. Hakker, J. Rosenbaum, B. Yamrom, Y. H. Lee, G. Narzisi, A. Leotta, J. Kendall, E. Grabowska, B. Ma, S. Marks, L. Rodgers, A. Stepansky, J. Troge, P. Andrews, M. Bekritsky, K. Pradhan, E. Ghiban, M. Kramer, J. Parla, R. Demeter, L. L. Fulton, R. S. Fulton, V. J. Magrini, K. Ye, J. C. Darnell, R. B. Darnell, E. R. Mardis, R. K. Wilson, M. C. Schatz, W. R. McCombie, M. Wigler *Neuron* **2012**, *74*, 285.
[55] Y. T. Shih, Y. P. Hsueh *Nat. Commun.* **2016**, *7*, 11020.
[56] Y. Sakai, C. A. Shaw, B. C. Dawson, D. V. Dugas, Z. Al-Mohtaseb, D. E. Hill, H. Y. Zoghbi *Sci. Transl. Med.* **2011**, *3*, 86ra49.
[57] Y. Nishimura, C. L. Martin, A. Vazquez-Lopez, S. J. Spence, A. I. Alvarez-Retuerto, M. Sigman, C. Steindler, S. Pellegrini, N. C. Schanen, S. T. Warren, D. H. Geschwind *Hum. Mol. Genet.* **2007**, *16*, 1682.
[58] A. G. Chiocchetti, D. Haslinger, M. Boesch, T. Karl, S. Wiemann, C. M. Freitag, F. Poustka, B. Scheibe, J. W. Bauer, H. Hintner, M. Breitenbach, J. Kellermann, F. Lottspeich, S. M. Klauck, L. Breitenbach-Koller *Mol. Autism* **2014**, *5*, 10.
[59] D. G. Amaral, B. A. Corbett U.S. Patent 7604948, **2009**.
[60] M. G. Butler, S. K. Rafi, A. M. Manzardo *Int. J. Mol. Sci.* **2015**, *16*, 6464.
[61] P. Berbel, D. Navarro, G. C. Roman *Front. Endocrinol.* **2014**, *5*, 146.
[62] B. M. Neale, Y. Kou, L. Liu, A. Ma'ayan, K. E. Samocha, A. Sabo, C. F. Lin, C. Stevens, L. S. Wang, V. Makarov, P. Polak, S. Yoon, J. Maguire, E. L. Crawford, N. G. Campbell, E. T. Geller, O. Valladares, C. Schafer, H. Liu, T. Zhao, G. Cai, J. Lihm, R. Dannenfelser, O. Jabado, Z. Peralta, U. Nagaswamy, D. Muzny, J. G. Reid, I. Newsham, Y. Wu, L. Lewis, Y. Han, B. F. Voight, E. Lim, E. Rossin, A. Kirby, J. Flannick, M. Fromer, K. Shakir, T. Fennell, K. Garimella, E. Banks, R. Poplin, S. Gabriel, M. DePristo, J. R. Wimbish, B. E. Boone, S. E. Levy, C. Betancur, S. Sunyaev, E. Boerwinkle, J. D. Buxbaum, E. H. Cook Jr., B. Devlin, R. A.

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Gibbs, K. Roeder, G. D. Schellenberg, J. S. Sutcliffe, M. J. Daly. *Nature* **2012**, *485*, 242.
[63] L. Liu, J. Lei, S. J. Sanders, A. J. Willsey, Y. Kou, A. E. Cicek, L. Klei, C. Lu, X. He, M. Li, R. A. Muhle, A. Ma'ayan, J. P. Noonan, N. Sestan, K. A. McFadden, M. W. State, J. D. Buxbaum, B. Devlin, K. Roeder *Mol. Autism* **2014**, *5*, 22.
[64] P. M. Kidd, *Altern. Med. Rev.* **2002**, *7*, 472.
[65] Y. Tejaswini, Y. A. Devi, Y. R. Ahuja, U. Araga, Q. Hasan *Int. J. Pharm. Sci. Res.* **2012**, *3*, 4989.
[66] J. P. Hussman, R. H. Chung, A. J. Griswold, J. M. Jaworski, D. Salyakina, D. Ma, I. Konidari, P. L. Whitehead, J. M. Vance, E. R. Martin, M. L. Cuccaro, J. R. Gilbert, J. L. Haines, M. A. Pericak-Vance *Mol. Autism* **2011**, *2*, 1.
[67] K. Wang, H. Zhang, D. Ma, M. Bucan, J. T. Glessner, B. S. Abrahams, D. Salyakina, M. Imielinski, J. P. Bradfield, P. M. Sleiman, C. E. Kim, C. Hou, E. Frackelton, R. Chiavacci, N. Takahashi, T. Sakurai, E. Rappaport, C. M. Lajonchere, J. Munson, A. Estes, O. Korvatska, J. Piven, L. I. Sonnenblick, A. I. Alvarez Retuerto, E. I. Herman, H. Dong, T. Hutman, M. Sigman, S. Ozonoff, A. Klin, T. Owley, J. A. Sweeney, C. W. Brune, R. M. Cantor, R. Bernier, J. R. Gilbert, M. L. Cuccaro, W. M. McMahon, J. Miller, M. W. State, T. H. Wassink, H. Coon, S. E. Levy, R. T. Schultz, J. I. Nurnberger, J. L. Haines, J. S. Sutcliffe, E. H. Cook, N. J. Minshew, J. D. Buxbaum, G. Dawson, S. F. Grant, D. H., Geschwind, M. A. Pericak-Vance, G. D. Schellenberg, H. Hakonarson *Nature* **2009**, *459*, 528.
[68] A. M. Persico, T. Bourgeron *Trends Neurosci.* **2006**, *29*, 349.
[69] C. Correia, G. Oliveira, A. M. Vicente *PLoS One* **2014**, *9*, e112399.
[70] D. Pinto, A. T. Pagnamenta, L. Klei, R. Anney, D. Merico, R. Regan, J. Conroy, T. R. Magalhaes, C. Correia, B. S. Abrahams, J. Almeida, E. Bacchelli, G. D. Bader, A. J. Bailey, G. Baird, A. Battaglia, T. Berney, N. Bolshakova, S. Bölte, P. F. Bolton, T. Bourgeron, S. Brennan, J. Brian, S. E. Bryson, A. R. Carson, G. Casallo, J. Casey, B. H. Chung, L. Cochrane, C. Corsello, E. L. Crawford, A. Crossett, C. Cytrynbaum, G. Dawson, M. de Jonge, R. Delorme, I. Drmic, E. Duketis, F. Duque, A. Estes, P. Farrar, B. A. Fernandez, S. E. Folstein, E. Fombonne, C. M. Freitag, J. Gilbert, C. Gillberg, J. T. Glessner, J. Goldberg, A. Green, J. Green, S. J. Guter, H. Hakonarson, E. A. Heron, M. Hill, R. Holt, J. L. Howe, G. Hughes, V. Hus, R. Igliozzi, C. Kim, S. M. Klauck, A. Kolevzon, O. Korvatska, V. Kustanovich, C. M. Lajonchere, J. A. Lamb, M. Laskawiec, M. Leboyer, A. Le Couteur, B. L. Leventhal, A. C. Lionel, X. Q. Liu, C. Lord, L. Lotspeich, S. C. Lund, E. Maestrini, W. Mahoney, C. Mantoulan, C. R. Marshall, H. McConachie, C. J. McDougle, J. McGrath, W. M. McMahon, A. Merikangas, O. Migita, N. J. Minshew, G. K. Mirza, J. Munson, S. F. Nelson, C. Noakes, A. Noor, G. Nygren, G. Oliveira, K. Papanikolaou, J. R. Parr, B. Parrini, T. Paton, A. Pickles, M. Pilorge, J. Piven, C. P. Ponting, D. J. Posey, A. Poustka, F. Poustka, A. Prasad, J. Ragoussis, K. Renshaw, J. Rickaby, W. Roberts, K. Roeder, B. Roge, M. L. Rutter, L. J. Bierut, J. P. Rice, J. Salt, K. Sansom, D. Sato, R. Segurado, A. F. Sequeira, L. Senman, N. Shah, V. C. Sheffield, L. Soorya, I. Sousa, O. Stein, N. Sykes, V. Stoppioni, C. Strawbridge, R. Tancredi, K. Tansey, B. Thiruvahindrapduram, A. P. Thompson, S. Thomson, A. Tryfon, J. Tsiantis, H. Van Engeland, J. B. Vincent, F. Volkmar, S. Wallace, K. Wang, Z. Wang, T. H. Wassink, C. Webber, R. Weksberg, K. Wing, K. Wittemeyer, S. Wood, J. Wu, B. L. Yaspan, D. Zurawiecki, L. Zwaigenbaum, J. D. Buxbaum, R. M. Cantor, E. H. Cook, H. Coon, M. L. Cuccaro, B. Devlin, S. Ennis, L. Gallagher, D. H. Geschwind, M. Gill, J. L. Haines, J. Hallmayer, J. Miller, A. P. Monaco, J. I.Jr. Nurnberger, A. D. Paterson, M. A. Pericak-Vance, G. D. Schellenberg, P. Szatmari, A. M. Vicente, V. J. Vieland, E. M. Wijsman, S. W. Scherer, J. S. Sutcliffe, C. Betancur *Nature* **2010**, *466*, 368.
[71] M. Bucan, B. S. Abrahams, K. Wang, J. T. Glessner, E. I. Herman, L. I. Sonnenblick, A. I. Alvarez Retuerto, M. Imielinski, D. Hadley, J. P. Bradfield, C. Kim, N. B. Gidaya, I. Lindquist, T. Hutman, M. Sigman, V. Kustanovich, C. M. Lajonchere, A. Singleton, J. Kim, T. H. Wassink, W. M. McMahon, T. Owley, J. A. Sweeney, H. Coon, J. I. Nurnberger, M. Li, R. M. Cantor, N. J. Minshew, J. S. Sutcliffe, E. H. Cook, G. Dawson, J. D. Buxbaum, S. F. Grant, G. D. Schellenberg, D. H. Geschwind, H. Hakonarson *PLoS Genet* **2009**, *5*, e1000536.
[72] T.-L. Lee, M. J. Raygada, O. M. Rennert *Gene* **2012**, *496*, 88.
[73] D. Pinto, E. Delaby, D. Merico, M. Barbosa, A. Merikangas, L. Klei, B. Thiruvahindrapuram, X. Xu, R. Ziman, Z. Wang, J. A. Vorstman, A. Thompson, R. Regan, M. Pilorge, G. Pellecchia, A. T. Pagnamenta, B. Oliveira, C. R. Marshall, T. R. Magalhaes, J. K. Lowe, J. L. Howe, A. J. Griswold, J. Gilbert, E. Duketis, B. A. Dombroski, M. V. De Jonge, M. Cuccaro, E. L. Crawford, C. T. Correia, J. Conroy, I. C. Conceição, A. G. Chiocchetti, J. P. Casey, G. Cai, C. Cabrol, N. Bolshakova, E. Bacchelli, R. Anney, S. Gallinger, M. Cotterchio, G. Casey, L. Zwaigenbaum, K. Wittemeyer, K. Wing, S. Wallace, H. van Engeland, A. Tryfon, S. Thomson, L. Soorya, B. Rogé, W. Roberts, F. Poustka, S. Mouga, N. Minshew, L. A. McInnes, S. G. McGrew, C. Lord, M. Leboyer, A. S. Le Couteur, A. Kolevzon, P. Jiménez González, S. Jacob, R. Holt, S. Guter, J. Green, A. Green, C. Gillberg, B. A. Fernandez, F. Duque, R. Delorme, G. Dawson, P. Chaste, C. Café, S. Brennan, T. Bourgeron, P. F. Bolton, S. Bölte, R. Bernier, G. Baird, A. J. Bailey, E. Anagnostou, J. Almeida, E. M. Wijsman, V. J. Vieland, A. M. Vicente, G. D. Schellenberg, M. Pericak-Vance, A. D. Paterson, J. R. Parr, G. Oliveira, J. I. Nurnberger, A. P. Monaco, E. Maestrini, S. M. Klauck, H. Hakonarson, J. L. Haines, D. H. Geschwind, C. M. Freitag, S. E. Folstein, S. Ennis, H. Coon, A. Battaglia, P. Szatmari, J. S. Sutcliffe, J. Hallmayer, M. Gill, E. H. Cook, J. D. Buxbaum, B. Devlin, L. Gallagher, C. Betancur, S. W. Scherer *Am. J. Hum. Genet.* **2014**, *94*, 677.
[74] S. R. Gilman, I. Iossifov, D. Levy, M. Ronemus, M. Wigler, D. Vitkup *Neuron* **2011**, *70*, 898.
[75] S. Sbacchi, F. Acquadro, I. Calò, F. Calì, V. Romano *Curr. Genom.* **2010**, *11*, 136.
[76] V. W. Hu, A. Nguyen, K. S. Kim, M. E. Steinberg, T. Sarachana, M. A. Scully, S. J. Soldin, T. Luu, N. H. Lee *PLoS One* **2009**, *4*, e5775.
[77] O. Penagarikano, B. S. Abrahams, E. I. Herman, K. D. Winden, A. Gdalyahu, H. Dong, L. I. Sonnenblick, R. Gruver, J. Almajano, A. Bragin, P. Golshani, J. T. Trachtenberg, E. Peles, D. H. Geschwind *Cell* **2011**, *147*, 235.
[78] H. B. Hutcheson, L. M. Olson, Y. Bradford, S. E. Folstein, S. L. Santangelo, J. S. Sutcliffe, J. L. Haines *BMC Med. Genet.* **2004**, *5*, 1.
[79] C. J. Carter *Pathog. Dis.* **2013**, *69*, 240.
[80] C. C. Quattrocchi, F. Wannenes, A. M. Persico, S. A. Ciafre, G. D'Arcangelo, M. G. Farace, F. Keller *J. Biol. Chem.* **2002**, *277*, 303.
[81] S. W. Kong, Y. Shimizu-Motohashi, M. G. Campbell, I. H. Lee, C. D. Collins, S. J. Brewster, I. A. Holm, L. Rappaport, I. S. Kohane, L. M. Kunkel *Neurogenetics* **2013**, *14*, 143.
[82] S. W. Kong, C. D. Collins, Y. Shimizu-Motohashi, I. A. Holm, M. G. Campbell, I. H. Lee, S. J. Brewster, E. Hanson, H. K. Harris, K. R. Lowe, A. Saada, A. Mora, K. Madison, R. Hundley, J. Egan, J. McCarthy, A. Eran, M. Galdzicki, L. Rappaport, L. M. Kunkel, I. S. Kohane *PLoS One* **2012**, *7*, e49475.
[83] H. Ivanov, V. Stoyanova, N. Popov, M. Bosheva, T. I. Vachev *BioDiscovery* **2015**, *17*, 2.
[84] P. Levitt, D. B. Campbell *The J. Clin. Invest.* **2009**, *119*, 747.
[85] A. Krishnan, R. Zhang, V. Yao, C. L. Theesfeld, A. K. Wong, A. Tadych, N. Volfovsky, A. Packer, A. Lash, O. G. Troyanskaya bioRxiv **2016**, https://doi.org/;10.1101/057828
[86] R. L. Stornetta, J. J. Zhu *Neuroscientist* **2011**, *17*, 54.
[87] J. Chen, I. Alberts, X. Li *Int. J. Dev. Neurosci.* **2014**, *35*, 35.
[88] J. N. Crawley, W. D. Heyer, J. M. LaSalle *Trends Genet.* **2016**, *32*, 139.
[89] D. H. Geschwind *Ann. Rev. Med.* **2009**, *60*, 367.
[90] M. L. Estes, A. K. McAllister *Nat. Rev. Neurosci.* **2015**, *16*, 469.

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[91] A. Faridar, D. Jones-Davis, E. Rider, J. Li, I. Gobius, L. Morcom, L. J. Richards, S. Sen, E. H. Sherr *Mol. Autism* **2014**, *5*, 1.
[92] C. Gottfried, V. Bambini-Junior, F. Francis, R. Riesgo, W. Savino *Front. Psychiatry* **2015**, *6*, 121.
[93] X. Li, A. Chauhan, A. M. Sheikh, S. Patil, V. Chauhan, X. M. Li, L. Ji, T. Brown, M. Malik *J. Neuroimmunol.* **2009**, *207*, 111.
[94] D. L. Vargas, C. Nascimbene, C. Krishnan, A. W. Zimmerman, C. A. Pardo *Ann. Neurol.* **2005**, *57*, 67.

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