Molecular Psychiatry (2007) 12, 292–306

www.nature.com/mp

ORIGINAL ARTICLE

A proteomic study of serum from children with autism showing differential expression of apolipoproteins and complement proteins

BA Corbett[1,2], AB Kantor[3], H Schulman[3], WL Walker[2,4], L Lit[2,4], P Ashwood[2,5], DM Rocke[6] and FR Sharp[2,4]

[1]*Department of Psychiatry and Behavioral Sciences, University of California at Davis, Sacramento, CA, USA;* [2]*Department of Psychiatry and Behavioral Sciences, MIND Institute, University of California at Davis, Sacramento, CA, USA;* [3]*PPD Biomarker Discovery Sciences, Menlo Park, CA, USA;* [4]*Department of Neurology, University of California at Davis, Sacramento, CA, USA;* [5]*Department of Medical Microbiology and Immunology, University of California at Davis, Sacramento, CA, USA and* [6]*Department of Public Health Sciences, Division of Biostatistics, University of California at Davis, Sacramento, CA, USA*

Modern methods that use systematic, quantitative and unbiased approaches are making it possible to discover proteins altered by a disease. To identify proteins that might be differentially expressed in autism, serum proteins from blood were subjected to trypsin digestion followed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) on time-of-flight (TOF) instruments to identify differentially expressed peptides. Children with autism 4–6 years of age ($n=69$) were compared to typically developing children ($n=35$) with similar age and gender distributions. A total of 6348 peptide components were quantified. Of these, five peptide components corresponding to four known proteins had an effect size >0.99 with a $P<0.05$ and a Mascot identification score of 30 or greater for autism compared to controls. The four proteins were: Apolipoprotein (apo) B-100, Complement Factor H Related Protein (FHR1), Complement C1q and Fibronectin 1 (FN1). In addition, apo B-100 and apo A-IV were higher in children with high compared to low functioning autism. Apos are involved in the transport of lipids, cholesterol and vitamin E. The complement system is involved in the lysis and removal of infectious organisms in blood, and may be involved in cellular apoptosis in brain. Despite limitations of the study, including the low fold changes and variable detection rates for the peptide components, the data support possible differences of circulating proteins in autism, and should help stimulate the continued search for causes and treatments of autism by examining peripheral blood.
Molecular Psychiatry (2007) **12**, 292–306. doi:10.1038/sj.mp.4001943; published online 26 December 2006

Keywords: apolipoprotein B-100; apolipoprotein; complement; autism; blood; proteomics

Introduction

Autism is a heterogeneous neurodevelopmental disorder characterized by impairments in social interactions, deficits in verbal and non-verbal communication, and repetitive and stereotyped patterns of behavior.[1,2] Although symptoms begin in infancy, diagnosis is dependent mainly on the recognition of the cardinal clinical signs that are present by 3 years of age.[3] Behavioral intervention is more effective the earlier it is initiated.[4–7] Therefore, the identification of biological markers of autism would have a number of ramifications: markers could assist in the early and accurate diagnosis of the disorder; they may identify particular biological targets for treatment; and they could be used to define subgroups for specific treatments as well as for genetic studies.

So far, no biological or genetic markers for autism have been identified. Autism is a complex disorder that could have a number of 'causes'. It has a strong genetic component with the concordance rate in monozygotic twins (60% autism, 90% broader phenotype) being much higher than that of dizygotic twins (3%); and the sibling recurrence risk ratio being five to 10 times that for the general population.[8–10] Although previous linkage studies have identified a number of potential genetic loci for autism, only a few of these regions have been confirmed.[11–17] No single gene or group of genes have been unequivocally linked to autism, leading to suggestions that as many as 15 genes may weakly interact to confer a susceptibility to the disorder.[18,19]

Among the genes implicated in autism, several relate to immune regulation and function, including HLA-antigen presentation molecules and components

Correspondence: Dr BA Corbett, Department of Psychiatry and Behavioral Sciences, MIND Institute, University of California, Davis, 2825 50th Street, Sacramento, CA 95817, USA.
E-mail: blythe.corbett@ucdmc.ucdavis.edu
Received 31 May 2006; revised 31 October 2006; accepted 6 November 2006; published online 26 December 2006

of the complement system.[14,20–27] Although abnormalities of cytokines and chemokines have been reported in the blood of patients with autism including plasma levels of tumor necrosis factor-α (TNF)-α, TNF receptor II, Interferon-gamma (IFNγ), Interleukin-12 (IL-12), and IL-10, no definitive profile has emerged. Indeed, the changes reported in cytokine levels may occur in separate subgroups of autism subjects that have different clinical phenotypes.[28–34] Other immune system alterations reported in subjects who have autism include the presence of autoantibodies in the blood directed to central nervous system (CNS) or brain antigens, and the presence of inflammation within the brain.[35–38] Finally, an increased frequency of autoimmune disorders has been described in the families of individuals with autism. Comi *et al*.[39] found a positive correlation between the risk of autism and the number of family members affected with an autoimmune disease, with the highest risk associated with maternal autoimmunity. Similarly, immune disorders such as asthma and allergies, as well as the autoimmune diseases diabetes and psoriasis, were increased in mothers of children with autism spectrum disorder (ASD).[40]

While these studies suggest that blood-borne molecules may be different in at least some phenotypes of autism, some studies had methodological problems or used small and heterogeneous samples of subjects. Moreover, relatively few molecules have been assayed. Thus, in order to more thoroughly examine potential markers, we have employed a proteomics approach with a relatively large and homogeneous population of children with autism. Proteomics uses a variety of different separation and mass spectrometry methods that enable the exploration of large numbers of serum proteins. Proteomics analysis has the advantage that it is relatively unbiased (except for its technical limitations), and does not make *a priori* assumptions about differences between sample groups. It is a powerful method that can generate new hypotheses by identifying candidate protein markers that may be important in disease pathogenesis and diagnosis.[41,42] Proteomics approaches complement genetic and genome initiatives and may elucidate the protein products that are associated with genetic abnormalities involved in complex neurodevelopmental disorders such as autism.[43] Several recent studies have demonstrated the power of proteomics in understanding disease processes and elucidating the molecular defects associated with various conditions.[42,44–47] Junaid *et al*.[48] recently performed a proteomics study of autopsied brains of individuals with autism and found abnormalities that led to the identification of a single nucleotide polymorphism (SNP) in glyoxalase I, suggesting that it could be a susceptibility factor in autism.[48]

The current cross-sectional study was designed to examine the proteome in serum from blood of children with autism ($n = 69$) ages four to six compared to typically developing children ($n = 35$) with similar age and gender distributions. Blood was drawn from children, the serum separated and subjected to column chromatography followed by mass spectrometry. The goal of this study was to identify a few of the most differentially expressed and most statistically significant peptides (and originating proteins) in the autism compared to control samples. A table of the data set will be included as a Supplementary Table.

Materials and methods

Study population

A total of 136 children between 4 and 6 years of age were enrolled in the study. Participants were recruited from the University of California at Davis MIND Institute Clinic and research database. Typically developing participants were recruited from area school districts and community centers.

The inclusion criteria for the autism group included a diagnosis of Autistic Disorder based on the DSM-IV criteria determined by an experienced neuropsychologist (BAC), which was further corroborated by the following measures. The *Autism Diagnostic Observation Schedule-Generic* (ADOS-G)[49] provides observation of a child's communication, reciprocal social interaction, and stereotyped behavior including an algorithm with cutoffs for autism and autism spectrum disorders. The *Autism Diagnostic Interview-Research* (ADI-R) is a comprehensive, semi-structured parent interview that assesses a child's developmental history and relevant behaviors characteristic of autism and generates a diagnostic algorithm for Autistic Disorder.[50] Children who did not meet full autism criteria including pervasive developmental disorder-not otherwise specified (PDD-NOS) and Asperger Syndrome were excluded from the study. The *Social Communication Questionnaire* (SCQ)[51] was used as a screening tool to ensure the absence of symptoms of autism in the typically developing control children. One child who had scores above the cutoff (score = 15) was excluded from the typically developing group, and was referred for further diagnostic evaluation.

The *Stanford-Binet Intelligence Scale*[52] is a standardized measure of cognitive functioning administered to all participants to provide a measure of overall intellectual ability (IQ). The broad average range is defined by a mean of 100 and a s.d. of 16. The autism group was further divided based on full scale IQ as follows: High-functioning autism (HFA) having an IQ $\geqslant 68$ and low-functioning autism (LFA) having an IQ < 68. Inclusion criteria for typically developing children (TYP) were: evidence of attaining normal developmental milestones; no diagnosis of autism or other developmental disabilities; and IQ $\geqslant 68$.

A comprehensive medical history form, the *AGRE Medical History Form* (AGRE), was given to the parents of all participants to provide demographic, medical and family history information. Subjects were excluded from the investigation if they had a

diagnosis of Fragile X or other serious neurological (e.g., seizures), psychiatric (e.g., bipolar disorder) or known medical conditions. All participants were screened via parental interview for current and past physical illness. Children with known endocrine, cardiovascular, pulmonary, liver, kidney or other medical disease were excluded from the study. Furthermore, failure to complete significant portions of the research protocol resulted in exclusion from the study. Twenty-two children were excluded due to failure to meet inclusion criteria, inability to balance across the group based on age or gender, or noncompliance with the protocol (e.g., unable to complete the blood draw). Further, the blood sample of one child with LFA was later determined to be unusable for proteomic analysis.

The final study group consisted of 104 children, 69 children with autism and 35 children in the typically developing group. Of the children with autism, 35 had HFA and 34 LFA. The children were chosen so that the age and gender distribution was similar in the three groups. The age range for the sample ranged from 4-years, 0-months to 6-years, 11-months. The gender ratio originally was the same across all three groups with 29 males and six females in each group. The loss of one sample due to missing Cysteine protection peaks in the MS data meant that the final composition of the LFA group was 29 males and five females. The HFA group had a mean age of 5.2 years and mean IQ of 79. The LFA group had a mean age of 5.5 years and a mean IQ of 55. The Typical group had a mean age of 5.7 years and an average IQ of 113. Descriptive statistics are presented in Table 1.

At the time of the investigation, the following medications were being used by the participants (as reported by the parents): allergy/asthma medication (Albuterol, Claritin, Nasonex, solvent: TYP 3, HFA 4, LFA 1); antipsychotic medications (Risperidone, Seroquel, Abilify: TYP 0, HFA 1, LFA 8); antidepressant medications (Paxil, Zoloft, Prozac, Celexa: TYP 0, HFA 2, LFA 4); attention deficit hyperactivity disorder medications (Strattera, Medidate, Ritalin: TYP 0, HFA 2, LFA 2); sleep medications (clonidine, melatonin: TYP 0, HFA 1, LFA 3); and, antiviral medication (Famvir: TYP 0, HFA 1, LFA 1). Some of the children were on several medications, including combinations of antipsychotic and antidepressant medications. To summarize, nine (of 35) children with HFA and 15 (of 34) children with LFA were on some medication. We cannot exclude the possibility that some of the differences in the groups were influenced by medications. However, this is an unavoidable complexity of studying children with a severe neurodevelopmental disorder like autism.

Participation in the study required two visits. During the first visit the ADIR, ADOS, Stanford Binet and parental interviews were performed. This session lasted approximately 3 $\frac{1}{2}$ h. The second visit consisted of a blood draw by a professional, pediatric phlebotomist at the MIND Institute. The University of California at Davis Internal Review Board (IRB) approved this study.

Sample collection procedures

Parents were asked to have their child fast prior to the blood draw, which required no consumption of food after midnight the evening before the collection. As part of the protocol, the family provided ongoing information regarding the health status of the child participant. The phlebotomy visit was rescheduled if the participant had been ill within the past 72 h or had an immunization within the past two weeks. The serum sample was collected in one 9.5 ml serum separator tube (BD Vacutainer SST tube, Becton Dickinson and Co; Franklin Lakes, NJ, USA). Samples were then sent chilled (on ice) to the proteomics lab (PPD, Menlo Park, CA, USA) and aliquots frozen within eight hours of blood draw. Lab personnel were blind to the diagnosis until after all samples were analyzed.

Mass spectrometry

These methods have been described in previous publications from PPD.[41,53–55] In brief, serum was fractioned into proteome and metabolome using a 5-kDa molecular weight cutoff spin filter (Millipore Corp., Bedford, MA, USA). Proteomic analysis was performed on serum proteins >5000 Da. After dilution with 25 mM PBS buffer (pH 6.0), affinity beads (Prometic Biosciences, Cambridge, UK) were applied

Table 1 Demographic information (age, gender and IQ) for the participants including: typically developing children (TYP), children with low functioning autism (LFA) and children with high functioning autism (HFA)

Group N	N	*Mean age*	*Gender*	*Full scale IQ*	*VIQ Verbal reasoning*	*PIQ Visual reasoning*
Typically developing (TYP)	35	5.7	29 male Six female	112.75 (6.32)	116.87 (8.84)	114.12 (11.39)
High functioning autism (HFA)	35	5.2	29 male Six female	78.17 (18.92)	78.27 (21.12)	84.54 (22.47)
Low functioning autism (LFA)	34	5.5	29 male Five female	55.43 (9.42)	59.42 (7.80)	62.31 (14.59)
Total	104	5.4	87 male 17 female			

The means and standard deviations are presented for Full Scale IQ, Verbal Reasoning (VIQ) and Visual Reasoning (PIQ).

to remove the six most abundant proteins that account for about 80–90% of the serum protein (albumin, IgG, IgA, haptoglobin, transferrin and antitrypsin). The remaining proteins were denatured, disulfide bond reduced, and sulfhydryl groups carboxymethylated before digestion by modified trypsin. The tryptic peptides were then profiled (individual molecules tracked across samples and their differential expression quantified) by reverse phase liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) on high-resolution ($R > 5000$) time-of-flight (TOF) instruments using a capillary chromatography column. The on-line chromatography used a water/acetonitrile 100-min gradient with 0.1% formic acid added to aid ionization efficiency and chromatographic behavior. Proprietary MassView software was used to track and quantify molecules for their differential expression.[55–57]

Identification of proteins was performed based upon the identification of peptides. Peptides of interest (significantly changing in expression level) were linked to tandem mass spectrometry (MS/MS) experiments on quadrupole TOF (Q-TOF) and ion-trap mass spectrometers using extra or similar sample material. The resulting MS/MS spectra contain fragmentation patterns with characteristic peptide backbone cleavages. Each MS/MS raw spectrum from an isolated precursor ion is compared using commercially available software with *in silico* protein digestion and fragmentation using NCBI's RefSeq database to find a match, and hence identification. A match-quality score was reported based on Mascot software from Matrix Science for the identification of peptides.[58]

Quantification strategy

We used an approach developed by PPD for quantification of LC-MS data, applicable to large numbers of proteins/peptides and metabolites for the purpose of differential expression measurements and discovery of biomarkers for example.[54–57] The differential quantification method used here relies on the changes in analyte signal intensities directly reflecting their concentrations in one sample relative to another. Samples are not mixed nor are the samples otherwise manipulated beyond that required for the LC-MS analysis itself. The sample preparation and LC-MS conditions need to be carefully controlled, however, for optimal results, and frequent quality control samples are analyzed to assure stable, reproducible performance.

This quantification technology employs overall spectral intensity normalization by employing signals of molecules that do not change concentration from sample to sample. In this way, a simple correction can be applied for any drift over time in overall LC-MS instrument response and/or differences in sample concentrations. PPD's proprietary MassView software performs normalization by determining the median of the ratios for a large number of molecular components, requiring no operator intervention. It also performs spectral smoothing, baseline subtraction, noise evaluation, isotopic analysis, peak identification, intensity evaluation, inter-scan evaluation to construct chromatographic peaks, inter-file (inter-sample) evaluation to establish molecular components for analysis, normalization (mentioned above), and quantification for the thousands of components (molecular ions corresponding to peptides).

Mass spectrometry data collection

Less than 1% of the samples were excluded from the statistical analyses because of technical difficulties. Specifically, data for one female LFA participant was invalid due to missing Cysteine protection peaks in the profile data (see patient data above).

Statistical methods

Statistical analyses were conducted to assess differences in proteomic variables. For the first comparison, all of the children with autism (HFA + LFA; $n = 69$) were compared to the typically developing, normal controls (TYP, $n = 35$) using a univariate mean group (unpaired) comparison test that was either parametric (unpaired *t*-test) or non-parametric (Wilcoxon–Mann–Whitney test) depending on the normality of the data. Additional comparisons used paired tests with even numbers of subjects per group. Pairing was based on run order and matching by age and gender and was intended to reduce variability. Similar to the group analysis, the children with high functioning autism (HFA, $n = 35$) and low functioning autism (LFA, $n = 34$) were compared to each other and to typical children (TYP, $n = 35$) using either parametric (paired *t*-test) or non-parametric (paired Wilcoxon test) tests depending on the normality of the data. Goodness-of-fit statistics (Shapiro–Wilk) and tests of skewness and kurtosis were performed to assess normality. Each peptide component was assigned a univariate *P*-value. $P < 0.05$ were selected as differentially expressed. The effect size is the difference of the means of the two groups divided by the standard deviation that is weighted by the number of subjects in each group. The effect size (along with the *P*-value) was used to determine whether the peptides differentiated the groups. An effect size of 0.5 is modest, 1.0 strong and 2.0 very strong. The effect size provides an index of the absolute difference between groups as well as the variance. For the purpose of this study, we have listed the peptides with an effect size of 0.99 or greater in the tables. Fold change, on the other hand, is the average of intensities in diagnostic groups divided by the average of intensities in the typical group without taking variability within the groups into account (for unpaired tests) and is the average of the intensity ratios for paired comparisons. In addition, we only included peptides with Mascot scores greater than 30.[58] It is important to note that the greater number of peptides that identify a given protein, the more confident one is in the validity of the data. Finally, we included proteins that met the aforementioned

criteria for which only one peptide was identified when other peptide components from the same protein gave consistent results, albeit with lower effect sizes or fold differences.

Results

Subjects

There were no statistical differences in ages among the three groups (TYP, HFA, LFA; $F(2,101) = 0.065$, $P = 0.80$). Table 1 shows the demographic information for the participants.

Proteomics

A large number of the peptides were identified using the PPD peptide library coupled with additional directed tandem mass spectrometry. There were 6348 peptide components quantified at an occurrence threshold (detection rate) of 25%, meaning that the component had to be detected and quantifiable in at least 25% of the samples. Each component is a distinct molecular ion and is based on the peak observed isotope. Of the total peptide components tracked, 61% (3895) had a Mascot score of 15 or better and were identified with 576 known proteins (Tables 2 and 3), (i.e., NCBI RefSeq accession numbers). Of these known proteins, 48% had two or more identified peptides.

Table 2 summarizes the findings for the comparison of children with autism to the typically developing children. Of the 6348 peptide components tracked, 243 were identified with a $P < 0.05$ (unpaired *t*-test) of which 151 were associated with known proteins. Similarly, Table 3 summarizes the findings for the comparison of the children with LFA compared to the children with HFA. In the comparison between LFA and HFA, of the 6348 peptide components tracked, 434 were identified with a $P < 0.05$ (paired *t*-test) of which 227 were known proteins. The data in both tables does not include any correction for multiple comparisons. When using multiple comparison correction (step-down Bonferroni *P*-value adjustment method of Holm[59,60] no significant proteins were observed. As a result of this, we followed up on the identified proteins with some additional tests.

Tables 2 and 3 also report the detection rate (DR) for the peptide components at each significance level. The DRs refers to the percentage of samples in which the peptide component could be detected. In samples that showed no value and were subsequently not available, it does not mean that the expression level was zero in the sample. Rather, the peptide could not be reliably detected in a given sample and beyond a given threshold. Generally, the DR did not differ among groups of subjects (medians HFA = 77, LFA, 71, TYP, 74). Differences in DR were $< 40\%$ for all components. Specific information can be found in the Tables and Supplementary material.

Because of the inability to find significant components utilizing multiple comparison corrections (i.e., Bonferroni–Holm, Benjamini–Hochberg), and the presence of missing values due to variable detection rate, this report focuses on the most expressed *identified* proteins with the highest effect size (0.99 or greater) and a *P*-value of < 0.05. Furthermore, for inclusion, the peptides needed to have a Mascot score of greater than 30.[58] The components without an ID are not included in the data or discussed here. All of the quantitative data in this study, however, including the components without an ID, will be provided in an Excel File to anyone interested in pursuing these results and will be supplied as supplemental tables to this report.

Table 2 Identified serum proteome peptides for children with Autism (Low functioning autism plus high functioning autism) compared to typically developing, normal controls

P-*value*	*Peptides*	*Number with ID*	*% DRs*
$P < 0.001$	5	3	41
$P < 0.005$	21	14	54
$P < 0.01$	46	30	61
$P < 0.05$	243	151	68
All	6348	3895	70

'Peptides' refers to the number of components identified. Number with ID is the number of peptides components with a Mascot score of 15 or more; and, % DRs (detection rates) is the percentage of the samples in which measured values were available because the particular component was detected in that sample.

Table 3 Identified serum proteome peptides for children with low functioning autism compared to children with high functioning autism

P-*value*	*Peptides*	*Number with ID*	*% DR*
$P < 0.001$	6	1	67
$P < 0.005$	36	14	69
$P < 0.01$	82	36	71
$P < 0.05$	434	227	69
All	6348	3895	69

'Peptides' refers to the number of components identified. Number with ID is the number of peptides with a Mascot score of 15 or more that correspond to known proteins; and, % DR (detection rate) is the percentage of the samples in which measured values were available because the particular component was detected in that sample.

Autism (all children with autism) compared with typically developing children

Using the above criteria, a total of five peptide components, that corresponded to four known proteins, were identified that were differentially expressed in serum of children with autism ($n = 69$) compared to typically developing children ($n = 35$) (Table 4). Note that the figures presented below (Figures 1–4) show the differences across all three groups even though the findings pertain to statistical

differences between autism (HFA and LFA combined) and typically developing children.

Table 4 Apolipoprotein (apo) B-100 precursor was significantly lower in serum of the children with autism compared to the typically developing controls (Figure 1, Table 4). This protein was identified by ten peptide components (Table 4). To further examine this relationship, we looked at the paired comparisons using the $P<0.05$ selection criteria alone. Using these relaxed statistical criteria, for the HFA vs TYP comparison, 21 peptide components out of 229 were differentially expressed between HFA children compared to those with typical development (Table 5). The number that should have been significant by chance was $(0.05)229=11.5$, and the two-sided statistical significance of having a number as large as 21 is $P=0.0058$. For the LFA vs TYP comparison, 37 peptides were differentially expressed (Table 6), with $P=3.6\times10^{-10}$. Almost all of these peptides had a downward trend (21 of 21 in Table 5; 34 of 37 in Table 6) (the chance of 21 in 21 being down randomly is $P=9.5\times10^{-7}$ (two-sided) and the chance of 34 in 37 being down randomly is $P=1.2\times10^{-7}$) and all had a Mascot score above 30 (except one in Table 5). These multiple peptides provide strong support for the differential expression of apo B-100 in autism (for both HFA and LFA).

A number of the differentially expressed proteins were related to the complement system (Figures 2–4; Table 4). Two of these proteins had only one peptide that met the full selection criteria (Table 4). One peptide component showed the C Chain precursor of the C1q subcomponent to be upregulated in autism versus typical controls (Figure 2; Table 4). Two other components for C1q showed the same trend, but did not meet the *P*-value selection criteria. One peptide component showed that Fibronectin 1 isoform 1 (out of 20 detected for this protein) was upregulated in autism versus typical controls (Figure 3; Table 4). For the other components for Fibronectin 1 isoform 1, however, 18 of 19 showed the same upward trend, but did not meet the *P*-value selection criteria. This result has a two-sided binomial *P*-value of $P=0.000040$.

Two different peptide components (out of three detected for this protein) met the criteria (effect size >0.99; $P<0.05$) for showing upregulation of Complement factor-H-related protein 1 precursor (FHR-1) in autism compared to typically developing children (Table 4). Figure 4, which uses one of the two different peptides from the FHR-1 protein, shows higher expression of FHR-1 in both the HFA and LFA subgroups compared to the typically developing children. It is important to emphasize that a number of other peptides were also detected related to the complement system ($P<0.05$), but are not shown in the Figures or Tables because they did not have an effect size of >0.99. These included peptides corresponding to FHR1, Fibronectin, complement component 4B proprotein and complement 3 precursor protein, and other complement proteins that are included in the Supplementary Tables to this report.

Table 4 Proteins/peptides regulated in the serum of children with autism compared to typically developing controls

Accession number	*Protein description*	*Peptide*	P-*value*	*Fold change*	*Effect Size*	*No. Peptides with P< 0.05*	*Mascot score*	*% DR TYP*	*% DR HFA*	*% DR LFA*
LPHUB	Apolipoprotein B-100 precursor	SEILAHWSPAK	0.001	−1.20	−1.06	10	74	54	60	57
Q03591	Complement factor H-related protein 1 precursor (FHR-1)	TGESAEFVCK	0.00005	1.33	1.33	2	63	43	43	47
Q03591	Complement factor H-related protein 1 precursor (FHR-1)	ITCTEEGWSPTPK	0.005	1.26	0.99	2	82	40	43	47
P02747	Complement C1q subcomponent, C chain precursor	FNAVLTNPQGDYDTSTGK	0.004	1.19	1.26	1	95	26	26	24
NP_002017.1	Fibronectin 1 isoform 1 preproprotein; cold-insoluble globulin	NLQPASEYTVSLVAIK	0.002	1.23	1.00	1	78	60	43	53

These proteins were significantly different in autism ($n=69$) compared to typical children ($n=35$) ($P\leqslant0.05$, unpaired *t*-test) and had an effect size of 0.99 or greater. Down (−) indicates lower levels in serum of children with autism compared to typical children. Peptide detection rate (DR) percentages are presented for all three groups.

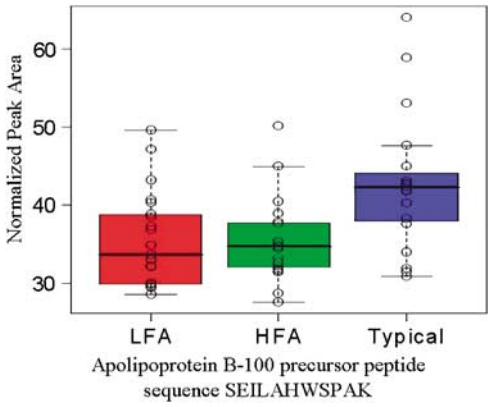

Figure 1 Apo B-100 (SEILAHWSPAK) concentrations in children with low functioning autism (LFA ■) and high functioning autism (HFA ■) compared to typical developing, normal children (typical ■. The peptide sequence used to identify this protein, the Accession Number, *P*-value and effect size are given in Table 4. The medians for the normalized peak areas for the three groups are: LFA 33.7, HFA 34.75, TYP 42.3; and the inter-quartile ranges are 9.01, 5.59 and 5.99, respectively.

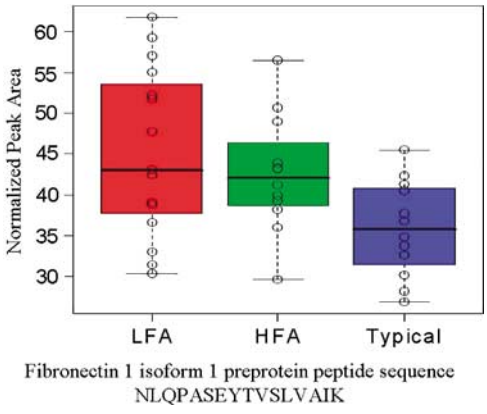

Figure 3 Fibronectin 1 (NLQPASEYTVSLVAIK) concentrations in children with low functioning autism (LFA ■) and high functioning autism (HFA ■) compared to typical developing, normal children (typical ■). The peptide sequence used to identify this protein, the Accession Number, *P*-value and effect size are given in Table 4. The medians for the normalized peak areas for the three groups are: LFA 43.0, HFA 42.09, TYP 35.74; and the inter-quartile ranges are 15.92, 6.26 and 8.72, respectively.

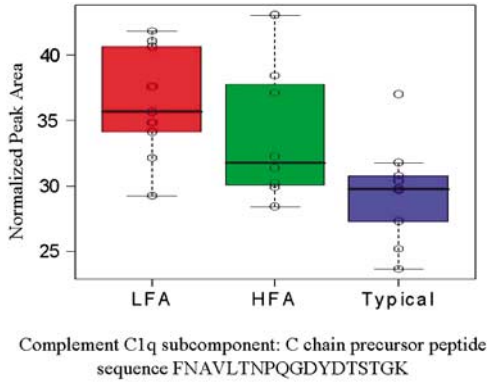

Figure 2 Complement C1q subcomponent: C chain (FNAVLTNPQGDYDTSTGK) concentrations in children with low functioning autism (LFA ■) and high functioning autism (HFA ■) compared to typical developing, normal children (typical ■). The peptide sequence used to identify this protein, the Accession Number, *P*-value and effect size are given in Table 4. The medians for the normalized peak areas for the three groups are: LFA 35.6, HFA 31.76, TYP 29.77; and the inter-quartile ranges are 6.5, 7.33 and 3.47, respectively.

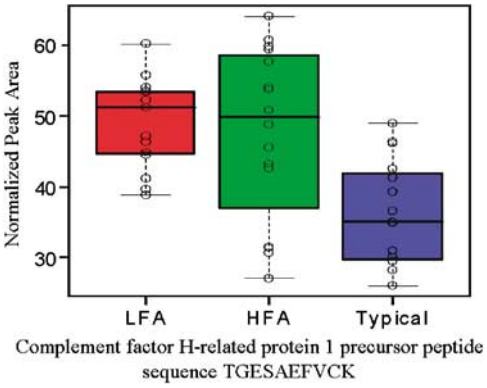

Figure 4 Complement factor H (TGESAEFVCK) concentrations in children with low functioning autism (LFA ■) and high functioning autism (HFA ■) compared to typical developing, normal children (typical ■). The peptide sequence used to identify this protein, the Accession number, *P*-value and the effect size are given in Table 4. The medians for the normalized peak areas for the three groups are: LFA 51.19, HFA 49.81, TYP 35.01; and the inter-quartile ranges are 8.8, 18.32 and 12.18, respectively. These findings were comparable to the other peptide ITC-TEEGWSPTPK that also identified FHR-1 (see Table 4).

Additional evidence that the differences in the complement proteins are real rather than statistical artifacts comes from the fact that three of the four proteins chosen according to the stringent criteria used for this study are from the complement system, suggesting a non-random concurrence.

Low functioning autism vs high functioning autism

Apo B-100 and apo A-IV were lower in the serum of the children with LFA compared with the children with HFA (Table 7). Apo B-100 was identified by nine peptides and Apo A-IV was identified by six peptides (Table 7; effect size >0.99 and $P<0.05$). As noted above, Apo B-100 is also decreased and on the list of proteins that differentiate all children with autism from typically developing children (Table 4).

A number of peptides related to the complement family were also differentially expressed in LFA vs HFA but did not meet the criteria to be included in Table 5. Details regarding these peptides are available as a supplement to this report. These peptides are

Table 5 The Apolipoprotein B-100 precursor peptide components that were significantly different in the serum of high functioning children with autism (HFA)

Accession number	*Protein description*	*Peptide*	*Mascot score*	*Fold change*	*Trend*	P *used*
LPHUB	Apolipoprotein B-100 precursor	SEILAHWSPAK	74	−1.25	Down	$P<0.001$
LPHUB	Apolipoprotein B-100 precursor	VNWEEEAASGLLTSLKDNVPK	68	−1.22	Down	$P<0.01$
LPHUB	Apolipoprotein B-100 precursor	IADFELPTIIVPEQTIEIPSIK	58	−1.12	Down	$P<0.01$
LPHUB	Apolipoprotein B-100 precursor	QTVNLQLQPYSLVTTLNSDLK	65	−1.09	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	YHWEHTGLTLR	49	−1.12	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	YSQPEDSLIPFFEITVPESQLTVSQFTLPK	45	−1.17	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	KGNVATEISTER	71	−1.09	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	LNGESNLR	35	−1.13	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	LEIQSQVDSQHVGHSVLTAK	55	−1.08	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	GSVLSR	37	−1.09	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	VLVDHFGYTK	53	−1.11	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	KADYVETVLDSTCSSTVQFLEYELNVLGTHK	25	−1.28	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SVSDGIAALDLNAVANK	110	−1.08	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	GIISALLVPPETEEAK	48	−1.12	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SNTVASLHTEK	59	−1.12	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SEYQADYESLR	66	−1.06	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SVSDGIAALDLNAVANK	110	−1.05	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	EVYGFNPEGK	75	−1.02	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SEYQADYESLR	66	−1.05	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	EYSGTIASEANTYLNSK	91	−1.08	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	YGMVAQVTQTLK	78	−1.23	Down	$P<0.05$

These peptides were significantly different between HFA ($n=35$) compared to typical children ($n=35$) ($P<0.05$, paired *t*-test). Effect size was not considered for selection of these components. Shading indicates levels of significance: darkest grey = $P<0.001$, dark grey = $P<0.005$, light grey = $P<0.01$, white = $P<0.05$.

Table 6 The Apolipoprotein B-100 precursor peptides that were significantly different in the serum of low functioning children with autism (LFA)

Accession number	*Protein description*	*Peptide*	*Mascot score*	*Fold change*	*Trend*	P *used*
LPHUB	Apolipoprotein B-100 precursor	SEILAHWSPAK	74	−1.20	Down	$P<0.001$
LPHUB	Apolipoprotein B-100 precursor	LELELRPTGEIEQYSVSATYELQR	72	−1.13	Down	$P<0.005$
LPHUB	Apolipoprotein B-100 precursor	SGSSTASWIQNVDTK	57	−1.21	Down	$P<0.005$
LPHUB	Apolipoprotein B-100 precursor	GIISALLVPPETEEAK	48	−1.16	Down	$P<0.005$
LPHUB	Apolipoprotein B-100 precursor	IADFELPTIIVPEQTIEIPSIK	58	−1.15	Down	$P<0.01$
LPHUB	Apolipoprotein B-100 precursor	LIVAMSSWLQK	61	−1.21	Down	$P<0.01$
LPHUB	Apolipoprotein B-100 precursor	IEGNLIFDPNNYLPK	64	−1.10	Down	$P<0.01$
LPHUB	Apolipoprotein B-100 precursor	NLQNNAEWVYQGAIR	98	−1.15	Down	$P<0.01$
LPHUB	Apolipoprotein B-100 precursor	VNWEEEAASGLLTSLKDNVPK	68	−1.21	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	VELEVPQLCSFILK	66	−1.10	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	VQGVEFSHR	57	1.34	Up	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	HSITNPLAVLCEFISQSIK	97	−1.14	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SNTVASLHTEK	59	−1.15	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SVSDGIAALDLNAVANK	110	−1.08	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	SLHMYANR	31	−1.13	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	LTISEQNIQR	77	1.30	Up	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	GSVLSR	37	−1.09	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	AVSMPSFSILGSDVR	110	−1.08	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	IEDGTLASK	62	−1.07	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	LNGESNLR	33	−1.11	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	QAEAVLK	49	−1.09	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	KLTISEQNIQR	87	−1.13	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	QTVNLQLQPYSLVTTLNSDLK	65	−1.06	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	KGNVATEISTER	71	−1.09	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	ATGVLYDYVNK	46	−1.06	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	FSVPAGIVIPSFQALTAR	70	−1.05	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	QHIEAIDVR	37	1.08	Up	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	ALYWVNGQVPDGVSK	62	−1.22	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	VELEVPQLCSFILK	66	−1.05	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	TSSFALNLPTLPEVK	66	−1.04	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	HSITNPLAVLCEFISQSIK	97	−1.05	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	ATLYALSHAVNNYHK	36	−1.22	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	FSVPAGIVIPSFQALTAR	70	−1.14	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	DKDQEVLLQTFLDDASPGDKR	89	−1.05	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	EVYGFNPEGK	75	−1.08	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	LAPGELTIIL	50	−1.04	Down	$P<0.05$
LPHUB	Apolipoprotein B-100 precursor	IADFELPTIIVPEQTIEIPSIK	58	−1.05	Down	$P<0.05$

These peptides were significantly different between LFA ($n=34$) compared to typical children ($n=35$) ($P\leqslant0.05$, paired *t*-test). Effect size was not considered for selection of these components. Shading indicates levels of significance: darkest grey = $P<0.001$, dark grey = $P<0.005$, light grey = $P<0.01$, white = $P<0.05$.

related to the following known proteins: complement C1r component precursor, complement component C9 precursor, complement component C8 beta chain precursor, clusterin isoform 1 or complement-associated protein SP-40, complement factor I precursor (C3B/C4B inactivator), Fibronectin precursor, and nuclear receptor co-activator 4 (NCoA-4) (70 kDa androgen receptor co-activator).

Discussion

The results support many previous studies that demonstrate differences of circulating immune related proteins in individuals with autism.[28,34] The current proteomic study has identified a number of circulating proteins that appear to be abnormal in autism compared with controls. Five peptide components showed the biggest differences between the Autism and the Typical groups, four of which correspond to three proteins involved in the complement pathway. Our data suggests peripheral differences of immune molecules including complement that could impact indirectly on the developing brain in autism. Alternatively, the same or similar molecules within the brain might be abnormal and contribute directly to abnormal brain development and autism.

Apolipoproteins

Apo are involved in the transport of lipids, cholesterol and vitamin E in the circulation and play a general role in the maintenance of lipid homeostasis. Apo B is a key structural component of several lipoproteins and is present in two forms: apo B (ApoB100) and

Table 7 Proteins/peptides significantly differentially expressed in the serum of children with autism with LFA compared to those with HFA

Accession number	*Protein description*	*Peptide*	P-*value*	*Fold change*	*Effect size*	*No. Peptides with P<0.05*	*Mascot score*	*% DR TYP*	*% DR HFA*	*% DR LFA*
LPHUB	Apolipoprotein B-100 precursor	IHSGSFQSQVELSNDQEK	0.032	−1.30	−1.14	9	60	54	60	57
NP_000473.1	Apolipoprotein A-IV precursor	LAPLAEDVR	0.014	−1.16	−1.12	6	69	37	54	43

These components were significantly different between LFA ($n = 34$) compared to HFA ($n = 34$) ($P \leqslant 0.05$, paired *t*-test) and had an effect size of 0.99 or greater. Down (−) indicates a decrease in serum of children with LFA compared to HFA. Peptide detection rate (DR) percentages are presented for all three groups.

ApoB48. ApoB100 is present on very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and LDL. ApoB100 assembles VLDL particles in the liver that starts formation of a pre-VLDL, which is retained in the cell unless converted to the triglyceride-poor VLDL2. Owing to its role in lipid transport and cholesterol homeostasis, it is not surprising that increased levels of ApoB100 plays a central role in the increased risk of vascular disease and the development of atherosclerosis.[61] Additionally, it has been reported that visceral obesity can lead to overproduction of ApoB100.[62]

The current data indicate a *reduction* in most peptide components for apo B-100, which differentiated the autism group as a whole from typical children as well as differentiating between LFA and HFA children. It is notable that overlapping as well as different peptides from the apo B-100 protein are lower in children with autism compared to controls (Table 4), HFA vs controls (Table 5), LFA versus controls (Table 6) and HFA vs LFA (Table 7). To complicate this even further, three of the 37 peptides regulated in the LFA vs Typical controls comparison show upregulation (Table 6). Though the explanation for these findings could be due to technical issues with proteomics, they could also relate to complex proteolytic cleavage of the whole apo B-100 protein. The data might suggest that apo B-100 protein is cleaved differently in HFA and LFA autism compared to controls, and perhaps some of the cleaved products of Apo B-100 proteins are differentially regulated so that most go down in autism but a few peptide components go up. Future studies will be required to sort out the explanation for these findings. The finding of increased apo E mRNA in the brains of subjects with autism[63] also shows up regulation of a different lipoprotein in autism.

It is unclear what the significance of the decrease in the apo is in the serum of the children with autism. It is possible that this indicates some abnormality of the gastrointestinal system, perhaps related to the immune abnormalities reported in the gastrointestinal tract in children with autism, that affects the apo.[29,31,64,65,66] Alternatively, the changes in apo could be due to differences in diet or various treatments.

Another possibility relates to the influence of the hypothalamic-pituitary-adrenocortical (HPA) system on lipid metabolism. Adrenocorticotropic hormone (ACTH) is a major component of the HPA axis and is involved in the regulation of lipid metabolism.[67] Children with autism demonstrate dysregulation of the HPA and are over-responsive to stress as shown by increases in cortisol.[68] ACTH directly and selectively decreases the expression and secretion of apo B *in vitro*,[69] hence possibly providing a link between stress and changes of ApoB-100.

As noted, two different apo were increased in the serum of children with HFA compared to LFA. Apo A-IV is a plasma lipoprotein synthesized mainly in small intestine enterocytes during fat absorption.[70] It

regulates lipid absorption, transport and metabolism[70] and may also act as a satiety signal.[71]

The finding that apo, especially ApoB 100, are reduced in children with autism warrants additional study to determine if it may play a role in CNS pathology, gastrointestinal function or even secondary factors, such as stress. As noted above, ApoE lipoprotein mRNA is *elevated* in the brain of individuals with autism,[63] perhaps suggesting a more generalized dysregulation of apometabolism in autism.

The complement system

Four of the seven major peptide components identified in this study correspond to proteins that are involved in the complement system, including increases in the C Chain precursor protein for complement factor-H related protein 1 precursor, complement C1q subcomponent, and fibronectin 1 isoform 1 preprotein. All of these proteins play a role in the complement system cascade, and are involved in complement activation pathways.[72–74]

As part of the innate immune system, the complement system acts as one of the first lines of defense for the lysis and removal of invading microbes. In addition, the complement system modulates the activation of B-lymphocytes and the production of antibodies, promotes chemotaxis and phagocyte recruitment, and is involved in the transport and clearance of immune complexes while preserving normal cells. Over 30 serum glycoprotein members of complement are activated in a tightly controlled, sequenced cascade. There are three main pathways that activate complement in response to an infection or foreign antigen: (1) the classical pathway – IgG and IgM driven antigen–antibody reactions; (2) the alternative pathway that is activated by polysaccharides from yeast, Gram-negative bacteria and other organisms and is independent of antibodies; and, (3) the lectin pathway (mannan-binding lectin – MBL pathway) that is activated when stimulated by mannose containing proteins found in viruses, yeasts and other organisms and is similar to the classical pathways but does not involve antibodies.[72] All three pathways activate the central C3 component.

Complement protein C1q and the classical pathway

C1q protein appears to be increased in serum of children with autism. C1q is intimately involved in the classical pathway of complement activation. C1q, the recognition subcomponent participates in the initial steps of the activation of the classic pathway and is involved in the MBL pathway.[74]

Phagocytosis of cellular targets, such as invading microbes and apoptotic cells, is enhanced if coated (opsonized) with complement including C1q.[75,76] Interestingly, increased deposition of C1q has been described in a group of autism patients with gastrointestinal symptoms, in which enhanced deposition of C1q was detected on intestinal epithelial cells and was co-localized with IgG.[77] This finding suggests that a C1q-antibody driven lytic response directed against a potential self-antigen in the intestinal cells, which could perturb the barrier functioning of the intestine, may be important in this subgroup of patients with autism. C1q not only plays a role in identifying cells for lysis, but also plays a role in the recruitment of inflammatory cells.[78,79] C1q knockout mice have a profound impairment of clearance of apoptotic cells.[80] This may be important in autism as neurons are notably sensitive to complement and specifically C1q mediated cell death.[81]

Fibronectin 1. Fibronectin proteins are also involved in the classical pathway and appear to be increased in serum of children with autism. Fibronectin 1 isoform (FN1, LETS) is a multifunctional extracellular matrix glycoprotein that participates in cell migration, signaling to adhering cells, apoptosis and in the complement system. Fibronectins bind a large number of molecules including fibrin, complement, collagen, heparin, proteoglycans and cells and bacteria.[82] One of the functions of fibronectin-1 is adhesion of cells to extracellular materials. Specifically, fibronectin binds to C1q and is involved in the clearance of C1q coated material like immune complexes, infectious agents and cellular debris.[82]

Factor H-related protein. Complement Factor H-related protein (FHR-1) appears to be increased in the serum of children with autism. Although the function of FHR-1 is not known, it is part of the multidomain, multifunctional Factor H gene family. Factor H is a plasma protein that plays an essential role in inhibiting complement activation in fluid phase and on cellular surfaces.[83] Factor H inhibits complement activation partly by inhibiting the BF-C3 complex in the alternate complement activation pathway. In addition, Factor H acts as an extracellular matrix component that binds to integrin receptors and interacts with various ligands.[84] FHR-1, the Factor H-like protein upregulated in the serum of children with autism in this study, is likely to have similar functions.

The factors that might produce these changes of complement are uncertain since upregulation of C1q and Fibronectin would activate complement, and the up regulation of FHR1 would inhibit complement. It is possible that one or more of these changes is due to genetic effects. For example, Odell *et al.*[14] evaluated 85 families with a child with autism compared to 69 control families. Nearly half (42.4%) of the subjects carried at least one C4BQ0 allele as compared with 14.5% of the controls ($P = 0.00013$, relative risk 4.33). More than half of the C4B null alleles in subjects with autism involved C4A duplications.[14] It is possible that a modest genetic abnormality of C4B might affect other complement proteins including fibronectin, C1q and FHR-1.

Should these changes of complement proteins be confirmed in future studies, it is unclear how

differences of various components of complement might contribute to autism. It is possible that the complement changes predispose to viral or other infections of mother or fetus during development, or to abnormal autoimmune responses, and that the ensuing immune response causes abnormalities of brain development that result in autism. Alternatively, it is possible that complement within the brain is abnormal and this directly contributes to abnormalities during neurodevelopment.

Role of complement in the brain

Complement plays a direct role within the nervous system, and is probably involved in cell death and apoptosis of cells during normal development and in CNS diseases like Alzheimer's, Huntington's disease, stroke and many others.[81,85] Most of the complement proteins found in blood are also expressed in the brain, mainly by microglia and to a lesser extent by stressed or damaged astrocytes and neurons.[81,86,87]

C1q, C3, C4, C5, C6, C9, CR1, C1qRp, CR3, CR4, Factor H and others have been shown to be upregulated in brain in humans and experimental animals with infection, ischemia and stroke, Alzheimer's disease, prion disease, Huntington's chorea and many other CNS diseases.[81,85,86] C1q-deficient mice have less injury following cerebral ischemia and have less neuropathology in transgenic mouse models of Alzheimer's disease.[88,89] Factor H is found in brain and may contribute to intracerebral complement system.[90] Fibronectin-deficient mice show increased neuronal apoptosis in brain.[91] Reelin (RELN) is an extracellular matrix protein that plays a pivotal role in the development of the CNS[92] and has been considered a candidate gene in autism.[10] Reelin, which must be secreted into the extracellular matrix to exert its biological effect,[93] degrades fibronectin[94] and hence could regulate fibronectin-C1q related removal of apoptotic cells in the nervous system.

With the alteration of complement proteins in serum of subjects with autism in this study, it is interesting to note a recent report of evidence of inflammation in the brains of individuals with autism.[38] Vargas *et al*.[38] found evidence of microglial activation in the cortex, subcortical white matter and the cerebellum of patients with autism consistent with a chronic and sustained neuroglial inflammatory response. It was suggested that activation of microglia could trigger complement activation that would contribute to brain injury.[38]

Limitations

Although the proteomic approach appears promising in the discovery of putative biological markers, the data also highlight the major difficulties facing previous and future studies. The small fold changes observed could indicate that there are specific subgroups that account for the changes detected in the group as a whole; or that the changes that occur in the serum are indeed small and that future replication will require quantitative and well-controlled studies.

A major challenge in proteomics pertains to the criteria used in the identification of proteins.[42] As there is a degree of uncertainty in the selection of peptides, we provided the Mascot score to provide some measure of confidence.[58] Thus, there is the concern of limited reproducibility and the need to unify and standardize the analytical methods.[47,95] Many or most monitored proteins are unanticipated at the time of study, eliminating the possibility of prior relative sensitivity factors. Additionally, methods based on introducing a known amount of a chemically analogous extraneous substance as an internal standard (i.e. 'spiking' of a standard reference material) are not practical.

In our results we include proteins for which only one peptide met the selection criteria when it was biologically related in a meaningful way to a protein with more than one peptide. However, we acknowledge that the greater number of peptides that identify a given protein the more confident one is in the validity of the data. The detection rate in some of the samples, such as C1q, is quite low and may simply represent a subpopulation of children with autism or extreme outliers. Further, proteins such as apo B-100 are highly abundant and the biological significance of small fold changes is unknown. Therefore, the data must be interpreted with caution. It will be important for future studies to include confirmatory analyses with alternative methods to support such findings.

Further, our analytic approach was based predominantly on statistical significance and selecting the proteins with the largest effect size for further analysis. The identification of other important proteins and peptides may show less statistical relevance, but ultimately reveal more biological importance in the study of autism. As autism is a heterogeneous disorder and these methods do not identify absolute values in the sample, future studies are clearly warranted to confirm and elucidate these findings.

Finally, since we are measuring peripheral blood, some of the abnormalities observed in the autism group may be due to a secondary factor, such as the individual's response to stress (venipuncture). Some children with autism respond to novel environmental stimuli by marked increases in salivary cortisol[68] that can also be detected in blood. It will be important for future studies to consider alternative explanations such as these when inferring CNS changes based on peripheral samples.

In summary, the proteomic approach used in this investigation succeeded in identifying a number of differentially expressed peptides that correspond to known proteins including apos and several involved in the complement cascade. This study demonstrates the potential of the proteomics approach, provides encouraging initial data, and should help stimulate the continued search for the causes and novel treatments of autism by examining peripheral blood.

Acknowledgments

We thank David G Amaral for the initial inspiration of this investigation. We are grateful to the many individuals at the MIND Institute especially Meridith Brandt, Anny Wu, Susan Bacalman, Veronica Lopez-Villasenor, Nuny Khamphay and many others that helped make the study possible. We are grateful to the individuals at PPD who contributed to sample processing and analysis, especially Hua Lin and Markus Anderle. Most importantly, we are indebted to the children and families who participated in this study. This study was supported by a grant from the MIND Institute, NIH grants to Frank R Sharp (NS043252; NS028167; and NS044283), David Rocke (P42-ES04699 and R01-HG003352) and by an NIH career development award to Blythe A Corbett (5K08NMHO72958).

References

1 Lord C, Cook EH, Leventhal BL, Amaral DG. Autism spectrum disorders. *Neuron* 2000; **28**: 355–363.
2 Lord C, Risi S, Lambrecht L, Cook Jr EH, Leventhal BL, DiLavore PC *et al.* The autism diagnostic observation schedule-generic: a standard measure of social and communication deficits associated with the spectrum of autism. *J Autism Dev Disord* 2000; **30**: 205–223.
3 Filipek PA, Accardo PJ, Ashwal S, Baranek GT, Cook Jr EH, Dawson G *et al.* Practice parameter: screening and diagnosis of autism: report of the Quality Standards Subcommittee of the American Academy of Neurology and the Child Neurology Society. *Neurology* 2000; **55**: 468–479.
4 McConnell SR. Interventions to facilitate social interaction for young children with autism: review of available research and recommendations for educational intervention and future research. *J Autism Dev Disord* 2002; **32**: 351–372.
5 Butter EM, Wynn J, Mulick JA. Early intervention critical to autism treatment. *Pediatr Ann* 2003; **32**: 677–684.
6 Ozonoff S, Goodlin-Jones BL, Solomon M. Evidence-based assessment of autism spectrum disorders in children and adolescents. *J Clin Child Adolesc Psychol* 2005; **34**: 541–547.
7 Francis K. Autism interventions: a critical update. *Dev Med Child Neurol* 2005; **47**: 493–499.
8 Bailey A, Phillips W, Rutter M. Autism: towards an integration of clinical, genetic, neuropsychological, and neurobiological perspectives. *J Child Psychol Psychiatry* 1996; **37**: 89–126.
9 Folstein SE, Rosen-Sheidley B. Genetics of autism: complex aetiology for a heterogeneous disorder. *Nat Rev Genet* 2001; **2**: 943–955.
10 Skaar DA, Shao Y, Haines JL, Stenger JE, Jaworski J, Martin ER *et al.* Analysis of the RELN gene as a genetic risk factor for autism. *Mol Psychiatry* 2005; **10**: 563–571.
11 Cantor RM, Kono N, Duvall JA, Alvarez-Retuerto A, Stone JL, Alarcon M *et al.* Replication of autism linkage: fine-mapping peak at 17q21. *Am J Hum Genet* 2005; **76**: 1050–1056.
12 Geschwind DH, Sowinski J, Lord C, Iversen P, Shestack J, Jones P *et al.* The autism genetic resource exchange: a resource for the study of autism and related neuropsychiatric conditions. *Am J Hum Genet* 2001; **69**: 463–466.
13 McCauley JL, Li C, Jiang L, Olson LM, Crockett G, Gainer K *et al.* Genome-wide and Ordered-Subset linkage analyses provide support for autism loci on 17q and 19p with evidence of phenotypic and interlocus genetic correlates. *BMC Med Genet* 2005; **6**: 1.
14 Odell D, Maciulis A, Cutler A, Warren L, McMahon WM, Coon H *et al.* Confirmation of the association of the C4B null allelle in autism. *Hum Immunol* 2005; **66**: 140–145.
15 Segurado R, Conroy J, Meally E, Fitzgerald M, Gill M, Gallagher L. Confirmation of association between autism and the mitochondrial aspartate/glutamate carrier SLC25A12 gene on chromosome 2q31. *Am J Psychiatry* 2005; **162**: 2182–2184.
16 Vorstman JA, Staal WG, van Daalen E, van Engeland H, Hochstenbach PF, Franke L. Identification of novel autism candidate regions through analysis of reported cytogenetic abnormalities associated with autism. *Mol Psychiatry* 2006; **11**: 18–28.
17 Wassink TH, Brzustowicz LM, Bartlett CW, Szatmari P. The search for autism disease genes. *Ment Retard Dev Disabil Res Rev* 2004; **10**: 272–283.
18 Risch N, Spiker D, Lotspeich L, Nouri N, Hinds D, Hallmayer J *et al.* A genomic screen of autism: evidence for a multilocus etiology. *Am J Hum Genet* 1999; **65**: 493–507.
19 Risch NJ. Searching for genetic determinants in the new millennium. *Nature* 2000; **405**: 847–856.
20 Ferrante P, Saresella M, Guerini FR, Marzorati M, Musetti MC, Cazzullo AG. Significant association of HLA A2-DR11 with CD4 naive decrease in autistic children. *Biomed Pharmacother* 2003; **57**: 372–374.
21 Purcell AE, Jeon OH, Pevsner J. The abnormal regulation of gene expression in autistic brain tissue. *J Autism Dev Disord* 2001; **31**: 545–549.
22 Torres AR, Maciulis A, Odell D. The association of MHC genes with autism. *Front Biosci* 2001; **6**: D936–D943.
23 Torres AR, Maciulis A, Stubbs EG, Cutler A, Odell D. The transmission disequilibrium test suggests that HLA-DR4 and DR13 are linked to autism spectrum disorder. *Hum Immunol* 2002; **63**: 311–316.
24 van Gent T, Heijnen CJ, Treffers PD. Autism and the immune system. *J Child Psychol Psychiatry* 1997; **38**: 337–349.
25 Warren RP, Odell JD, Warren WL, Burger RA, Maciulis A, Daniels WW *et al.* Strong association of the third hypervariable region of HLA-DR beta 1 with autism. *J Neuroimmunol* 1996; **67**: 97–102.
26 Warren RP, Yonk J, Burger RW, Odell D, Warren WL. DR-positive T cells in autism: association with decreased plasma levels of the complement C4B protein. *Neuropsychobiology* 1995; **31**: 53–57.
27 Warren RP, Singh VK, Averett RE, Odell JD, Maciulis A, Burger RA *et al.* Immunogenetic studies in autism and related disorders. *Mol Chem Neuropathol* 1996; **28**: 77–81.
28 Ashwood P, Van de Water J. A review of autism and the immune response. *Clin Dev Immunol* 2004; **11**: 165–174.
29 DeFelice ML, Ruchelli ED, Markowitz JE, Strogatz M, Reddy KP, Kadivar K *et al.* Intestinal cytokines in children with pervasive developmental disorders. *Am J Gastroenterol* 2003; **98**: 1777–1782.
30 Jyonouchi H, Sun S, Le H. Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. *J Neuroimmunol* 2001; **120**: 170–179.
31 Jyonouchi H, Geng L, Ruby A, Reddy C, Zimmerman-Bier B. Evaluation of an association between gastrointestinal symptoms and cytokine production against common dietary proteins in children with autism spectrum disorders. *J Pediatr* 2005; **146**: 605–610.
32 Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, Dienger K, Manning-Courtney P *et al.* Elevated cytokine levels in children with autism spectrum disorder. *J Neuroimmunol* 2006; **14**: 198–205.
33 Singh VK. Plasma increase of interleukin-12 and interferon-gamma. Pathological significance in autism. *J Neuroimmunol* 1996; **66**: 143–145.
34 Zimmerman AW, Jyonouchi H, Comi AM, Connors SL, Milstien S, Varsou A *et al.* Cerebrospinal fluid and serum markers of inflammation in autism. *Pediatr Neurol* 2005; **33**: 195–201.
35 Ashwood P, Van de Water J. Is autism an autoimmune disease? *Autoimmun Rev* 2004; **3**: 557–562.
36 Silva SC, Correia C, Fesel C, Barreto M, Coutinho AM, Marques C *et al.* Autoantibody repertoires to brain tissue in autism nuclear families. *J Neuroimmunol* 2004; **152**: 176–182.
37 Singh VK, Rivas WH. Prevalence of serum antibodies to caudate nucleus in autistic children. *Neurosci Lett* 2004; **355**: 53–56.
38 Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann Neurol* 2005; **57**: 67–81.

39 Comi AM, Zimmerman AW, Frye VH, Law PA, Peeden JN. Familial clustering of autoimmune disorders and evaluation of medical risk factors in autism. *J Child Neurol* 1999; **14**: 388–394.
40 Croen LA, Grether JK, Yoshida CK, Odouli R, Van de Water J. Maternal autoimmune diseases, asthma and allergies, and childhood autism spectrum disorders: a case-control study. *Arch Pediatr Adolesc Med* 2005; **159**: 151–157.
41 Kantor AB. Comprehensive phenotyping and biological marker discovery. *Dis Markers* 2002; **18**: 91–97.
42 Shen Y, Kim J, Strittmatter EF, Jacobs JM, Camp II DG, Fang R *et al.* Characterization of the human blood plasma proteome. *Proteomics* 2005; **5**: 4034–4045.
43 Junaid MA, Pullarkat RK. Proteomic approach for the elucidation of biological defects in autism. *J Autism Dev Disord* 2001; **31**: 557–560.
44 Banks RE, Dunn MJ, Hochstrasser DF, Sanchez JC, Blackstock W, Pappin DJ *et al.* Proteomics: new perspectives, new biomedical opportunities. *Lancet* 2000; **356**: 1749–1756.
45 Chan SM, Utz PJ. The challenge of analyzing the proteome in humans with autoimmune diseases. *Ann NY Acad Sci* 2005; **1062**: 61–68.
46 Kantor AB, Alters SE, Cheal K, Dietz LJ. Immune systems biology: immunoprofiling of cells and molecules. *Biotechniques* 2004; **36**: 520–524.
47 Xiao Z, Prieto D, Conrads TP, Veenstra TD, Issaq HJ. Proteomic patterns: their potential for disease diagnosis. *Mol Cell Endocrinol* 2005; **230**: 95–106.
48 Junaid MA, Kowal D, Barua M, Pullarkat PS, Sklower Brooks S, Pullarkat RK. Proteomic studies identified a single nucleotide polymorphism in glyoxalase I as autism susceptibility factor. *Am J Med Genet A* 2004; **131**: 11–17.
49 Lord C, Rutter M, DiLavore PC, Risi S. *Autism Diagnostic Observation Schedule (ADOS)*. Western Psychological Services: Los Angeles, CA, 2002.
50 Lord C, Rutter M, Le Couteur A. Autism diagnostic interview-revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *J Autism Dev Disord* 1994; **24**: 659–685.
51 Berument SK, Rutter M, Lord C, Pickles A, Bailey A. Autism screening questionnaire: diagnostic validity. *Br J Psychiatry* 1999; **175**: 444–451.
52 Thorndike RL, Hagen EP, Sattler JM. *The Stanford Binet Intelligence Scale*, 4th edn. Riverside Publishing: Chicago, 1986.
53 O'Connor KC, Roy S, Becker C, Hafler DA, Kantor AB. Comprehensive phenotyping in multiple sclerosis: discovery based proteomics and the current understanding of putative biomarkers. *Disease Mark* 2006; **22**: 213–225.
54 Roy SM, Becker CH. Quantification of proteins and metabolites by mass spectrometry without isotopic labeling. In: Sechi S (ed). *Methods in Molecular Biology,* Vol 359. *Quantitative Proteomics by Mass Spectrometry*. Humana Press: Totowa, NJ, 2007, pp 87–105.
55 Wang W, Zhou H, Lin H, Roy S, Shaler TA, Hill LR *et al.* Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. *Anal Chem* 2003; **75**: 4818–4826.
56 Becker C, Hastings CA, Norton S, inventors. Mass spectrometric quantification of chmical mixture components. USA patent 6,835,927, 2002.
57 Hastings CA, Norton SM, Roy S. New algorithms for processing and peak detection in liquid chromatography/mass spectrometry data. *Rapid Commun Mass Spectrom* 2002; **16**: 462–467.
58 Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999; **20**: 3551–3567.
59 Blair RC, Troendle JF, Beck RW. Control of familywise errors in multiple endpoint assessments via stepwise permutation tests. *Statis Med* 1996; **15**: 1107–1121.
60 Holm S. A simple sequentially rejective multiple test procedure. *Scand J Statist* 1979; **6**: 65–70.
61 Olofsson SO, Boren J. Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. *J Intern Med* 2005; **258**: 395–410.
62 Marsh JB. Lipoprotein metabolism in obesity and diabetes: insights from stable isotope kinetic studies in humans. *Nutr Rev* 2003; **61**: 363–375.
63 Purcell AE, Jeon OH, Zimmerman AW, Blue ME, Pevsner J. Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology* 2001; **57**: 1618–1628.
64 Ashwood P, Anthony A, Torrente F, Wakefield AJ. Spontaneous mucosal lymphocyte cytokine profiles in children with autism and gastrointestinal symptoms: mucosal immune activation and reduced counter regulatory interleukin-10. *J Clin Immunol* 2004; **24**: 664–673.
65 Erickson CA, Stigler KA, Corkins MR, Posey DJ, Fitzgerald JF, McDougle CJ. Gastrointestinal factors in autistic disorder: a critical review. *J Autism Dev Disord* 2005; **35**: 713–727.
66 Jyonouchi H, Sun S, Itokazu N. Innate immunity associated with inflammatory responses and cytokine production against common dietary proteins in patients with autism spectrum disorder. *Neuropsychobiology* 2002; **46**: 76–84.
67 Alfano J, Pedersen RC, Kramer RE, Brownie AC. Cholesterol metabolism in the rat adrenal cortex: acute temporal changes following stress. *Can J Biochem Cell Biol* 1983; **61**: 708–713.
68 Corbett BA, Mendoza S, Abdullah M, Wegelin JA, Levine S. Cortisol circadian rhythms and response to stress in children with autism. *Psychoneuroendocrinology* 2006; **31**: 59–68.
69 Xu N, Ekstrom U, Nilsson-Ehle P. ACTH decreases the expression and secretion of apolipoprotein B in HepG2 cell cultures. *J Biol Chem* 2001; **276**: 38680–38684.
70 Stan S, Delvin E, Lambert M, Seidman E, Levy E. Apo A-IV: an update on regulation and physiologic functions. *Biochim Biophys Acta* 2003; **1631**: 177–187.
71 Qin X, Tso P. The role of apolipoprotein AIV on the control of food intake. *Curr Drug Targets* 2005; **6**: 145–151.
72 Parkin J, Cohen B. An overview of the immune system. *Lancet* 2001; **357**: 1777–1789.
73 Barrington R, Zhang M, Fischer M, Carroll MC. The role of complement in inflammation and adaptive immunity. *Immunol Rev* 2001; **180**: 5–15.
74 Tosi MF. Innate immune responses to infection. *J Allergy Clin Immunol* 2005; **116**: 241–249.
75 Korb LC, Ahearn JM. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J Immunol* 1997; **158**: 4525–4528.
76 Webster SD, Park M, Fonseca MI, Tenner AJ. Structural and functional evidence for microglial expression of C1qR(P), the C1q receptor that enhances phagocytosis. *J Leukoc Biol* 2000; **67**: 109–116.
77 Torrente F, Ashwood P, Day R, Machado N, Furlano RI, Anthony A *et al.* Small intestinal enteropathy with epithelial IgG and complement deposition in children with regressive autism. *Mol Psychiatry* 2002; **7**: 375–382, 334.
78 van den Berg RH, Faber-Krol MC, Sim RB, Daha MR. The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells. *J Immunol* 1998; **161**: 6924–6930.
79 Ogden CA, Elkon KB. Role of complement and other innate immune mechanisms in the removal of apoptotic cells. *Curr Dir Autoimmun* 2006; **9**: 120–142.
80 Botto M, Walport MJ. C1q, autoimmunity and apoptosis. *Immunobiology* 2002; **205**: 395–406.
81 Francis K, Van Beek J, Canova C, Neal JW, Gasque P. Innate immunity and brain inflammation: the key role of complement. *Expert Rev Mol Med* 2003; **2003**: 1–19.
82 Bing DH, Almeda S, Isliker H, Lahav J, Hynes RO. Fibronectin binds to the C1q component of complement. *Proc Natl Acad Sci USA* 1982; **79**: 4198–4201.
83 Rodriguez de Cordoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sanchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol* 2004; **41**: 355–367.
84 Zipfel PF. Complement factor H: physiology and pathophysiology. *Semin Thromb Hemost* 2001; **27**: 191–199.

85 van Beek J, Elward K, Gasque P. Activation of complement in the central nervous system: roles in neurodegeneration and neuroprotection. *Ann NY Acad Sci* 2003; **992**: 56–71.
86 Tuppo EE, Arias HR. The role of inflammation in Alzheimer's disease. *Int J Biochem Cell Biol* 2005; **37**: 289–305.
87 van Beek J, van Meurs M, t Hart BA, Brok HP, Neal JW, Chatagner A *et al.* Decay-accelerating factor (CD55) is expressed by neurons in response to chronic but not acute autoimmune central nervous system inflammation associated with complement activation. *J Immunol* 2005; **174**: 2353–2365.
88 Ten VS, Sosunov SA, Mazer SP, Stark RI, Caspersen C, Sughrue ME *et al.* C1q-deficiency is neuroprotective against hypoxic-ischemic brain injury in neonatal mice. *Stroke* 2005; **36**: 2244–2250, (E-pub 22 September 2005).
89 Fonseca MI, Zhou J, Botto M, Tenner AJ. Absence of C1q leads to less neuropathology in transgenic mouse models of Alzheimer's disease. *J Neurosci* 2004; **24**: 6457–6465.
90 Serrano J, Encinas JM, Fernandez AP, Castro-Blanco S, Alonso D, Fernandez-Vizarra P *et al.* Distribution of immunoreactivity for the adrenomedullin binding protein, complement factor H, in the rat brain. *Neuroscience* 2003; **116**: 947–962.
91 Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA, Wieloch T *et al.* Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. *Nat Med* 2001; **7**: 324–330.
92 Fatemi SH. Reelin glycoprotein: structure, biology and roles in health and disease. *Mol Psychiatry* 2005; **10**: 251–257.
93 D'Arcangelo G, Nakajima K, Miyata T, Ogawa M, Mikoshiba K, Curran T. Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J Neurosci* 1997; **17**: 23–31.
94 Quattrocchi CC, Wannenes F, Persico AM, Ciafre SA, D'Arcangelo G, Farace MG *et al.* Reelin is a serine protease of the extracellular matrix. *J Biol Chem* 2002; **277**: 303–309.
95 Baggerly KA, Morris JS, Coombes KR. Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments. *Bioinformatics* 2004; **20**: 777–785.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)