A Pilot Proteomic Analysis of Salivary Biomarkers in Autism Spectrum Disorder

Armand G. Ngounou Wetie, Kelly L. Wormwood, Stefanie Russell, Jeanne P. Ryan, Costel C. Darie, and Alisa G. Woods

Autism spectrum disorder (ASD) prevalence is increasing, with current estimates at 1/68–1/50 individuals diagnosed with an ASD. Diagnosis is based on behavioral assessments. Early diagnosis and intervention is known to greatly improve functional outcomes in people with ASD. Diagnosis, treatment monitoring and prognosis of ASD symptoms could be facilitated with biomarkers to complement behavioral assessments. Mass spectrometry (MS) based proteomics may help reveal biomarkers for ASD. In this pilot study, we have analyzed the salivary proteome in individuals with ASD compared to neurotypical control subjects, using MS-based proteomics. Our goal is to optimize methods for salivary proteomic biomarker discovery and to identify initial putative biomarkers in people with ASDs. The salivary proteome is virtually unstudied in ASD, and saliva could provide an easily accessible biomaterial for analysis. Using nano liquid chromatography-tandem mass spectrometry, we found statistically significant differences in several salivary proteins, including elevated prolactin-inducible protein, lactotransferrin, Ig kappa chain C region, Ig gamma-1 chain C region, Ig lambda-2 chain C regions, neutrophil elastase, polymeric immunoglobulin receptor and deleted in malignant brain tumors 1. Our results indicate that this is an effective method for identification of salivary protein biomarkers, support the concept that immune system and gastrointestinal disturbances may be present in individuals with ASDs and point toward the need for larger studies in behaviorally-characterized individuals. ***Autism Res*** *2015, 8: 338–350.* © 2015 International Society for Autism Research, Wiley Periodicals, Inc.

Keywords: ASD; proteomics; biomarker signature; early detection; diagnosis

Introduction

Autism spectrum disorder (ASD) has an estimated prevalence of about 1 in 68 US children, 1 in 42 boys [Wingate et al., 2014]. According to a recent survey study, as many as 1 in 50 children may have ASD [Blumberg et al., 2013]. Unfortunately, causes of ASD are incompletely understood and ASD in children is often undiagnosed [Dawson, 2010; Zwaigenbaum, 2010]. Diagnosis can be missed at any age, even in adults [Van Schalkwyk et al., in press]. Current screening tools for ASD are based on behavior, and can generate false positive identification [Dereu et al., 2012]. Treatments for ASD have been shown in many studies to be most effective when initiated early [Lovaas, 1987; Eldevik et al., 2009; Eikeseth et al., 2012], therefore, diagnosis of ASD at as early an age as possible is imperative. Biomarkers for ASD could potentially aid in early ASD detection, detection at any age, and also may help to elucidate ASD subtypes.

Biomarkers are being increasingly used to predict or identify illnesses, disorders, and other physiological differences in many fields, including in the fields of psychiatry and neurodevelopmental disorders [Aubert et al., 2013; Walker et al., 2013; Zoladz & Diamond, 2013]. According to the United States Food and Drug Administration, a biomarker is an objective measurement of a normal or pathologic biological process or an objective measurement indicating treatment response [Katz, 2004]. There are many genomic studies focusing on ASDs, with over 1,000 implicated ASD-associated genes [Conciatori et al., 2004; Hu et al., 2009; Freitag et al., 2010; State & Levitt, 2011]. Based on these studies, ASD likely has multiple causes and subtypes [O'Roak et al., 2012]. A recent study indicated genetic association of the CHD8 mutation with an autism subtype, the providing the clearest genetic association with an ASD to date [Bernier et al., 2014]. Further study of the protein products resulting from genetic mutations, or even environmental influences is, however, needed.

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Abbreviations: MS, mass spectrometry; NanoLC-MS/MS, nano liquid chromatography-tandem mass spectrometry; TIC, total ion current; *m/z*, mass/charge; CID, collision-induced dissociation; ASD, Autism spectrum disorder

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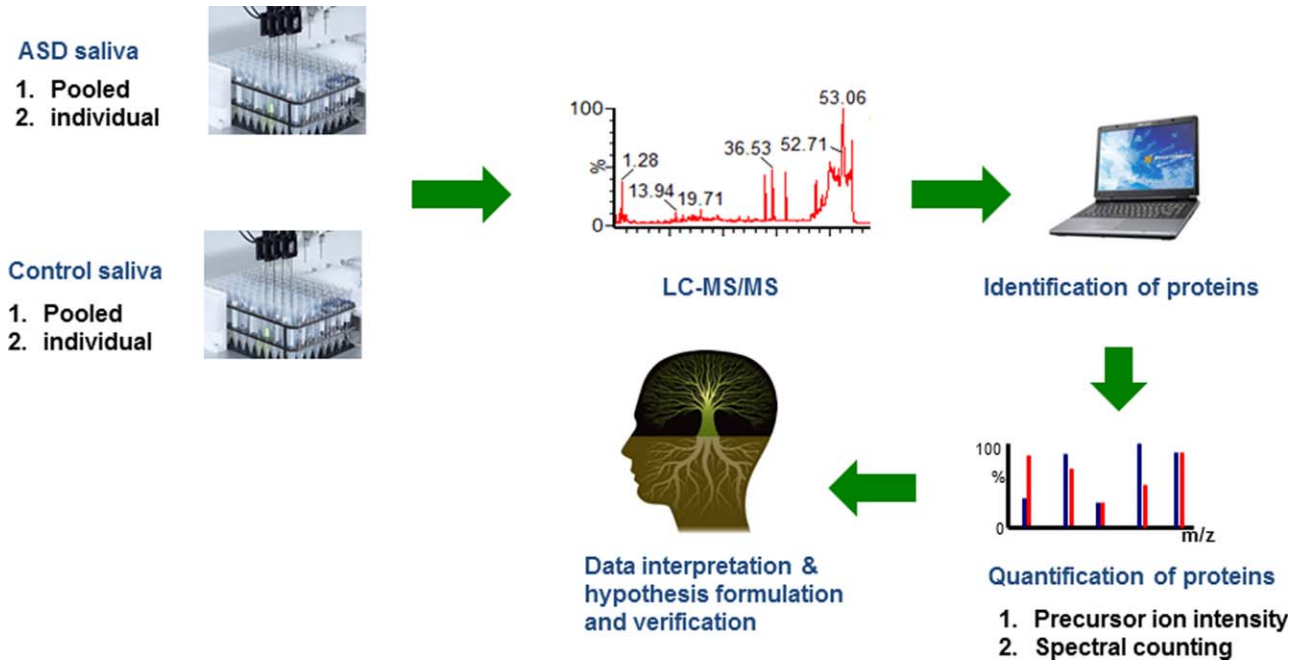

Figure 1. Representation of the strategy utilized in this study. Pooled and individual saliva samples from both diseased and healthy subjects are processed by in-solution tryptic digestion followed by nanoLC-MS/MS for identification of dysregulated proteins.

Unlike genetic studies, proteomic studies in ASDs are relatively scant. Recent developments in biochemistry technology could help facilitate study of ASD at the protein level, providing much-needed insights into causes and consequences [Broek et al., 2014; Ngounou Wetie, Sokolowska, et al., 2014]. Convergence of multiple genetic pathways to affect a few common protein biomarkers has been proposed for Alzheimer's disease, and it is conceivable that such protein convergence may exist in ASD [Talwar et al., 2014], warranting further study of ASD at the proteomic level.

Because proteins are the active effectors of biological processes, study of proteins could help elucidate the functional deficits found in ASDs and give clues to etiology. Protein biomarkers may ultimately aid in diagnosis, prognosis, and treatment monitoring in ASDs, and we may be able to understand the common pathways generally found in specific neurodevelopmental disorders through study of protein biomarkers [Sokolowska et al., in press; Woods et al., 2013]. The main current technique used in proteomics is mass spectrometry (MS) [Woods et al., 2012a, b, 2013]. MS can reliably and comprehensively identify putative protein biomarkers [Darie et al., 2011; Ngounou Wetie et al., 2013a, b, c; Ngounou Wetie, Wormwood, et al., 2014; Sokolowska et al., 2011, 2012, 2013; Woods et al., 2012a, b]. MS is indeed being used for newborn screening for several disorders, including phenylketonuria, congenital hypothyroidism, and cystic fibrosis [Wilcken, 2012].

Using MS, putative biomarkers have been identified in human blood sera from people with ASDs. These include alterations in complement proteins, which are part of an immune system response [Corbett et al., 2007; Momeni, Bergquist, et al. 2012; Momeni, Brudin, et al. 2012)], and in apolipoproteins, which are cholesterol-carrying proteins [Corbett et al., 2007; Momeni, Bergquist, et al., 2012; Momeni, Brudin, et al., 2012; Woods et al., 2012a, b; Ngounou Wetie, Wormwood, et al., 2014]. Reinforcing the concept that lipid disturbances may be present in ASD, a recent MS study found that females with Asperger's syndrome (an ASD subtype) had differences in proteins related to lipid transport and metabolism relative to controls. Interestingly, males predominantly demonstrated differences in proteins related to inflammation [Steeb et al., 2014].

We have recently reported a pilot proteomic analysis of blood serum taken from children with ASD, compared to matched control participants. Consistent with the idea that cholesterol disturbances may be present in some forms of ASD, we found increased levels of apolipoproteins (apos) apoA1 and apoA4 and of serum paraoxanase/arylesterase 1 (PON1) in ASD sera compared to controls. All three proteins are predicted to interact and are parts of high density lipoproteins [Ngounou Wetie, Wormwood, et al., 2014].

Studies of blood sera have provided putative ASD biomarkers, however, saliva could be another source of human biomaterials that may be more accessible to researchers and clinicians, as collection of saliva does

Table 1. Subject Demographics

Description of the study cohort						
Subject #	Diagnosis	Gender	Age	Language use	Comorbidities	Medication
A1	Autism	M	12	Verbal, mild to moderate	ADHD, anxiety	Strattera, citalopram
A2	Autism	M	16	Severe delays in functioning, language	ADHD, behavioral disturbances	Risperidone, Concerta, Sertraline
A3	Autism	M	8	Verbal, mild to moderate	Allergies	Claritin, multivitamin
A4	PDD-NOS	M	13	Verbal, mild to moderate	Epilepsy	Lamictal
A6	Autism, possible Asperger's	M	10	High functioning, verbal	None reported	None
A7	Autism	M	11	Verbal, mild to moderate	None reported	Multivitamin
B1	none	M	9	N/A	None	None
B2	none	M	6	N/A	None	None
B3	none	M	13	N/A	None	None
B4	none	M	10	N/A	None	None
B5	none	M	11	N/A	None	None
B6	none	M	8	N/A	None	None

Note. A5 was removed due to an insufficient sample.

not require a blood draw, a licensed medical professional and saliva collection is less aversive to study participants compared to blood collection. Saliva contains at least 2,290 proteins, compared to approximately 2,698 proteins found in blood plasma, making it a rich source of potential biomarkers [Loo et al., 2010]. However, only one published study has undertaken proteomic analysis of saliva in idiopathic autism, reporting that several salivary proteins undergo hypophosphorylation in ASDs, including statherin, histatin 1, and acidic proline-rich proteins [Castagnola et al., 2008].

We initiated this study to optimize methods for saliva biomarker analysis in ASD, using MS-based proteomics. An overview work-flow of the strategy adopted in this whole study is presented in Figure 1. We report significantly different levels of several salivary proteins in individuals with ASD compared to neurotypical control subjects, including elevated prolactin-inducible protein (PIP), lactotransferrin (LTF), Ig kappa chain C region, Ig gamma-1 chain C region, Ig lambda-2 chain C regions, neutrophil elastase, polymeric immunoglobulin receptor, and deleted in malignant brain tumors 1. We found significantly elevated statherin in controls, and elevated histatin 1 and salivary acidic proline rich protein in controls at levels approaching significance. This pilot study supports the possibility that immunological responses are present in some forms of ASD, and could provide a first step toward a diagnostic test for ASD. Further validation and measurement of these putative biomarkers in larger subject numbers is needed.

Experimental Design

Sample Collection

Human saliva samples were collected at the State University of New York Neuropsychology clinic under IRB approval and participant identities were unknown to the investigators conducting MS. Subject demographics are found in Table 1. Investigators conducting MS were unaware of whether saliva contained samples from individuals with ASD or were control samples (blind analysis). Saliva was collected via passive drool into a straw and collection cup. Participants (ages 5–17) either had DSM-IV-TR-diagnosed ASD or were control subjects without any ASD diagnosis or diagnosis of any other neurodevelopmental, psychiatric, or major medical condition. Approximately 1–2 mL of saliva was collected then spun for 20 min in a centrifuge to pellet/remove debris, and the supernatant was removed and placed in microtubes. Saliva was then immediately frozen at -20°C and stored until MS analyses.

Sample Preparation

Saliva samples from 6 donors (100 μg of protein per sample as determined by a Bradford protein assay) were analyzed twofold. In the first case, 6 individual saliva samples from 6 donors were pooled in one ASD sample (ASD pooled sample) and the same was done with the 6 control samples (Control pooled sample). In the other case, samples were processed individually (i.e., without pooling). Concretely; for the pooled sample analysis, samples A1–A6 (ASD) were pooled into one sample (A) whereas B1–B6 (Control) was pooled into another sample (B). These same samples (A1–A6 and B1–B6) were the ones that were analyzed individually. Samples were subjected to buffer exchange in high pressure liquid chromatography (HPLC) grade water using PD-10 columns and then concentration with a 3kDa-MWCO Centricon concentrator (Millipore) followed by centrifugal evaporation in a Speedvac concentrator. The samples were then resuspended in 100 μL of 80% (v/v) Acetonitrile (ACN), 50 mM Trizma-HCl and 10 mM $CaCl_2$ at pH 7.6 and sonicated for 15–20 min in a water

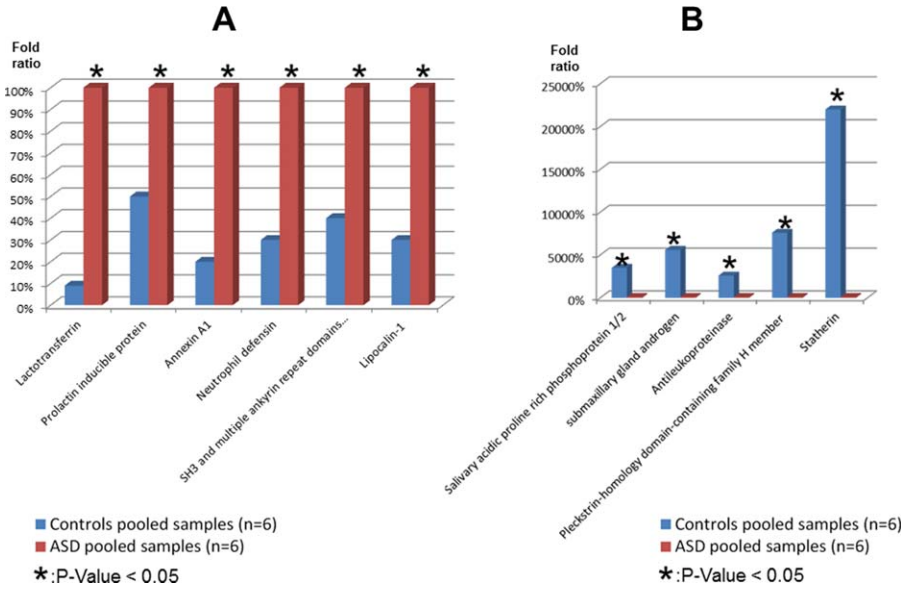

Figure 2. Identification of dysregulated proteins within the pooled saliva of ASD and controls. The individual saliva samples from 6 donors were pooled in one ASD sample (ASD pooled sample), digested by trypsin and analyzed by LC-MS/MS in triplicates. The control samples from 6 donors identically (Control pooled samples). The raw data was processed (as described in the materials and methods section) and then uploaded onto Scaffold 4.0 software and analyzed for the relative quantitation using the ratio between the normalized spectral counts (levels of proteins) for each protein within the ASD pooled sample and control pooled sample. The proteins that had a significantly different distribution with ASD versus control samples (and a different ratio) were also investigated for statistical significance, as determined by the Fisher's exact test using the *P*-Value (within the Scaffold 4.0 software). Here, only the proteins with a *P*-Value < 0.05 are shown. A: proteins with lower levels in ASD, as compared with controls. B: proteins with higher levels in ASD, as compared with controls.

bath. The disulfide bonds were reduced by incubation with 10 mM DTT for 60 min at 60°C and the reduced cysteine residues were alkylated by incubation with 20 mM iodoacetamide in the dark at room temperature for 60 min. Proteolysis with trypsin was performed for 16 h at 37°C, with a protein to trypsin ratio of 50:1. The trypsin solution was prepared in 50 mM Trizma-HCl, 10 mM $CaCl_2$ pH 7.6 at a concentration of 20 ng/μL. The digestion was performed in the same buffer. The digestion was then stopped by addition of formic acid (FA) to a final concentration of 2% FA. The samples were then centrifuged for 30 min at 14,000 rpm to remove insoluble material and the soluble peptide mixtures were then dried by a Speedvac and resolubilized in 10 μL of buffer A (98% H_2O, 2% ACN, and 0.1% FA) for LC-MS/MS analysis.

MS and Protein Identification

The resulting peptide mixture was analyzed by a nanoliquid chromatography-tandem mass spectrometer (nanoLC-MS/MS) using a NanoAcquity UPLC (Waters Corp., Milford, MA) coupled to a QTOF Micro MS (Micromass/Waters, Milford, MA) through a 20 μm ID picotip emitter (New Objective, Woburn, MA). Briefly, the peptides were loaded onto a C18 1.7 μm, 150 μm × 100 mm reversed phase column (Waters Corp.) and eluted either over a 60 or 150 min gradient of 2–100% ACN in 0.1% FA at a flow rate of 400 nL/min. The mass spectrometer was operated in the data-dependent mode and automatically switched between MS and MS/MS. MS data acquisition involved survey MS scans and automatic data-dependent MS/MS of 2+, 3+, and 4+ ions. The MS/MS was triggered when the MS signal intensity exceeded 10 counts per second. In survey MS scans, the four most intense peaks were selected for collision-induced dissociation and fragmented until the total MS/MS ion counts reached 5,000 or for up to 6 sec each. The full MS scan covered the *m/z* range from 400 to 1,350. Partial scanning ranges for increased time for the instrument to sequence the peptides (which would also lead to increased sequence coverage) were also used (e.g., *m/z* of 400–750, 700–1,050, and 1,000–1,350). Calibration of the mass spectrometer was performed for both precursor and product ions using 100 fmol GluFib standard peptide (Glu1-Fibrinopeptide B) with the amino acid sequence EGVNDNEEGFFSAR and a calculated mass for the monoisotopic peak of 1,770.68. The precursor ion monitored had an *m/z* of 785.84 (2+).

The raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software with the following parameters: background subtraction of polynomial order 5 adaptive with a threshold of 35%, two smoothings with a window of three channels in Savitzky–Golay mode, and

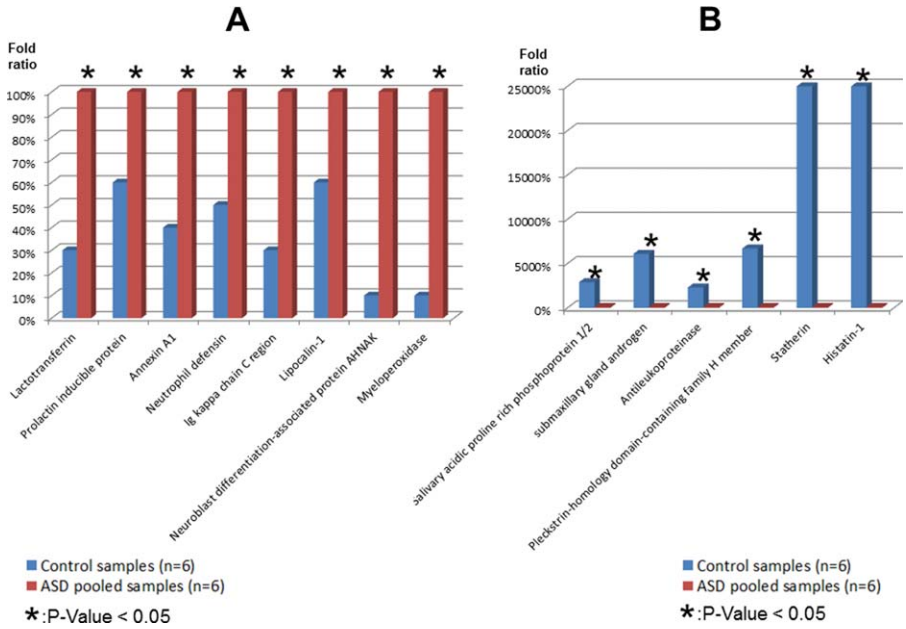

Figure 3. Identification of dysregulated proteins within the saliva of ASD and control samples. The individual saliva samples from 6 ASD and 6 control donors were digested by trypsin and analyzed by LC-MS/MS in triplicates. The raw data for ASD and control samples was processed and then uploaded into one ASD sample and one control sample onto Scaffold 4.0 software and analyzed for the relative quantitation using the ratio between the normalized spectral counts (levels of proteins) for each protein within the ASD and control samples. The proteins that had a significantly different distribution with ASD versus control samples (and a different ratio) were also investigated for statistical significance, as determined by the Fisher's exact test using the *P*-Value (within the Scaffold 4.0 software). Here, only the proteins with a *P*-Value < 0.05 are shown. A: proteins with lower levels in ASD, as compared with controls. B: Proteins with higher levels in ASD, as compared with controls.

centroid calculation of the top 80% of peaks based on a minimum peak width of 4 channels at half-height. The resulting pkl files were submitted to public database search for protein identification (www.matrixscience.com), using the following parameters: human databases from SwissProt (SwissProt_2013_09 database, selected for Homo sapiens, 20,272 entries); parent mass error, 1.3 Da; product ion error, 0.8 Da; enzyme used, trypsin; one missed cleavage; carbamidomethyl-cysteine as fixed modification and methionine oxidized as variable modification. In addition, acetylation of lysine and phosphorylation of serine, threonine, and tyrosine were also specified in Mascot as variable modifications, but this information was not used in the current manuscript. Further, dat-files were generated from the Mascot search results that were used for data processing using the Scaffold 4.0 software package. On this regard, the dat-files were combined in two categories or samples: ASD and controls. For the experiment presented in Figure 2 and Supporting Information Table 1, the dat-files for three technical replicates of the ASD pooled samples were combined in one ASD sample in Scaffold. The control samples were treated identically (three technical replicates combined). For the experiment presented in Figure 3 and Supporting Information Table 2, the dat-files for three technical replicates of the 6 ASD samples were combined in one ASD sample in Scaffold. The 6 control samples were treated identically: three technical replicates of the 6 control samples were combined in one control sample in Scaffold. For the experiment presented in Supporting Information Table 3, the dat-files for three technical replicates for each of the 6 ASD samples were combined into one ASD sample (there were a total of 6 ASD samples), which were also grouped into the ASD category. The control samples were treated the same. In summary, when we processed the data with scaffold, we combined both the samples run individually and the samples run as pooled into one category for the ASD subjects and into another category for the controls for comparison (Figs. 2 and 3; Supporting Information Tables 1 & 2). However, to account for individual subject variability, we also processed each of the 6 samples separately, that is, 6 different ASD categories and 6 different Control categories (Fig. 4; Supporting Information Table 3).

Criteria for Protein Identification

Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were

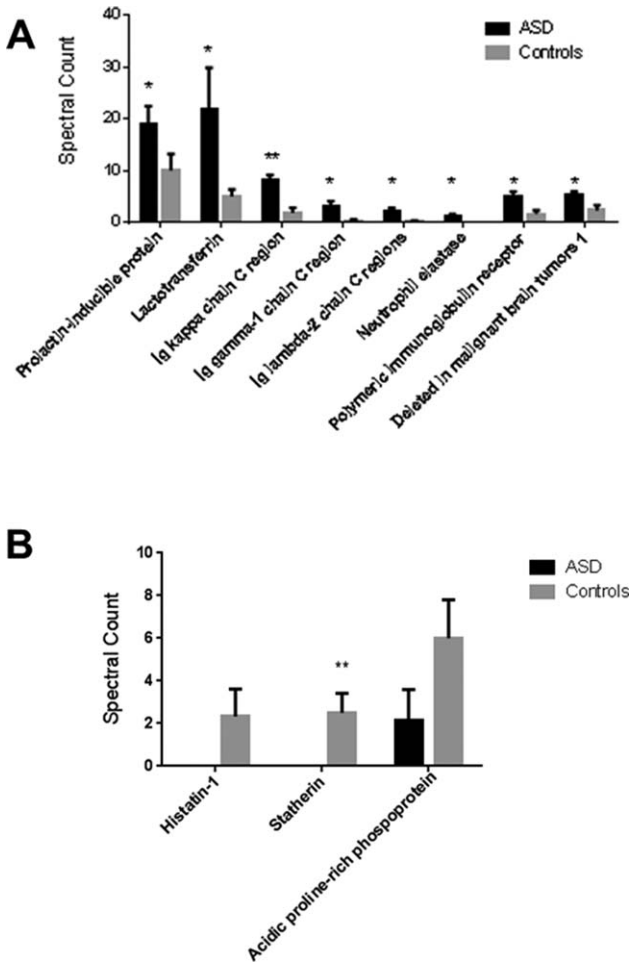

Figure 4. Relative quantitation of the salivary proteins, as determined by Scaffold 4.0 software and graphed using Prism Software (Graphpad, San Diego). The quantitation was performed based on the normalized spectral counts. Shown are graphs for the proteins that are more abundant in the saliva of ASD, as compared with the controls (A: PIP, LTF, Ig kappa chain C region, Ig lambda-2 chain C regions, neutrophil elastase, polymeric immunoglobulin receptor, deleted in malignant brain tumors 1, $*P<0.05$, $**P<0.005$) or less abundant in the saliva of ASD, as compared with the controls (B: histatin-1, statherin, acidic proline-rich phosphoprotein, $P<0.005$).

accepted if they could be established at greater than 20.0 % probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [Nesvizhskii et al., 2003]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were annotated with GO terms from NCBI (downloaded September 28, 2013) [Ashburner et al., 2000].

Quantitative Analysis of Dysregulated Proteins

Quantitation of differentially regulated proteins within each individual ASD and Control samples was performed in two ways: precursor ion intensity and spectral counting. For relative quantitation using the intensity of the precursor ion, we summed the extracted ion chromatograms for one precursor ion (with *m/z* of 873.30 (3+) which corresponded to a peptide that was part of Histatin-1) for 1 min, followed by direct comparison of the summed TOF MS spectra (for this precursor ion) from each of the 6 ASD and 6 control samples (through direct linking of the vertical axes, which allowed for the same intensity scale in all 12 samples). With regard to spectral counting, protein quantitation was performed by comparing the ASD and control categories using the normalized spectral counts option within the Scaffold software. For normalization, Scaffold sums the total spectrum counts for each run over the list of proteins. Then, the average among the different sums is calculated and scaled so that they all amount to the average. The scaling factor for each sample is then applied to each protein group and its total count is adjusted to a quantitative value. The outcome was a ratio between the proteins within the two ASD and control categories. The statistical analysis was also investigated for each protein using the Fisher's exact test and individual comparisons were performed using the Student's *t* test. *P*-Values of 0.05 or lower were considered statistically significant.

Protein–Protein Interaction Analysis

Identification of the interaction partners for our proteins of interest was performed using the freely-available STRING software (http://string-db.org/newstring_cgi/show_input_page.pl), using instructions provided in the published work [Szklarczyk et al., 2011; Franceschini et al., 2013].

Results

In Solution Digestion and Nanolc-MS/MS: Pooled Samples ASD Versus Pooled Samples Controls

When looking for biomarkers, it is always helpful to identify more general markers that can be used to identify a disease or a disorder in any individual. A common approach in cancer biomarker research is to pool samples to amplify the possibility of finding markers for quick initial screening, and to examine whether further study should be pursued [Chen et al., 2013; Mu et al., 2013]. As such, keeping in mind that ASD is likely a heterogeneous disorder [Talkowski et al., 2014], we initially sought to investigate the differences between pooled saliva ASD and control samples as an efficient first pass at uncovering biomarkers. To do so, we pooled

the saliva samples from 6 individuals with ASD and 6 matched controls, digested them with trypsin, ran them by nanoL C-MS/MS and compared the outcome of the database search to identify the dysregulated proteins. The outcome is shown in Figure 2 and Supporting Information Table 1. We identified a large number of proteins as well as prominent differences between proteins identified in the saliva of children with ASD and their matched controls. For example, we found increased levels of PIP (50%), LTF (9%) annexin A1 (20%), neutrophil-defensin 1 (30%), lactoperoxidase (50%), or lipocalin-1 (30%) in ASD versus controls and decreased levels of salivary acidic proline-rich phosphoprotein 1/2 (3,500%), submaxillary gland androgen-regulated protein 3B (5,600%), antileukoproteinase (2,600%), pleckstrin-homology domain-containing family H member (7,600%) or statherin (22,000%) in ASD versus controls. Using Fisher's exact test, all of these proteins were significantly different between the two groups (*P*-value lower than 0.05), except antileukoproteinase (*P*-value of 0.092) and lactoperoxidase (*P*-value of 0.10). Therefore, our results suggest dysregulated proteins are present in the pooled ASD saliva samples as compared with pooled matched controls, which can be identified by nanoLC-MS/MS analysis.

Comparison of the Pooled ASD and Control Samples to the Individual Samples

To validate and compare the results of our pooled analysis, we next investigated by nanoLC-MS/MS the individual ASD and control saliva samples and then numerically pooled them into two categories: ASD and controls. Again, we identified a large number of proteins as well as prominent differences between proteins identified in the saliva of children with ASD and their matched controls. For example, we found the same proteins that were dysregulated when we analyzed the pooled samples (Fig. 3 and Supporting Information Table 2), but also additional dysregulated proteins. Specifically, increased levels of PIP (59%), LTF (30%), annexin A1 (40%), neutrophil-defensin 1 (50%), lactoperoxidase (71%), or lipocalin-1 (59%), polymeric immunoglobulin receptor (40%), deleted in malignant brain tumors 1 protein (59%), IgG gamma-1 chain C region (30%), IgG kappa chain C region (30%) or myeloperoxidase (10%) in ASD versus controls. We further found decreased levels of salivary acidic proline-rich phosphoprotein 1/2 (2,900%), submaxillary gland androgen-regulated protein 3B (6,100%), antileukoproteinase (2,300%), statherin (INF-fold), pleckstrin-homology domain-containing family H member (6,700%) and histatin (INF-fold) in ASD as compared with controls. Using Fisher's exact test, almost all of these proteins were significantly different between the two groups (*P*-value lower than 0.05), except pleckstrin-homology domain-containing family H member, which approached statistical significance (*P*-value of 0.056), and two other proteins that were not statistically significant (lactoperoxidase with a *P*-value of 0.18 and polymeric immunoglobulin receptor with a *P*-value of 0.15). Therefore, most of the proteins found dysregulated in the pooled samples were also found in the individual samples when analyzed grouped into categories, with a similar ratio and backed by statistical significance.

Dysregulated Proteins Found in Our Initial Screening are Confirmed When Analyzed as Individual Samples

Combining many experimental technical and/or biological replicates of ASD and controls into two categories is useful in the initial stages of screening samples for identification of the initial biomarker candidates that are more likely to be found in any ASD sample, as compared with the controls. However, ideally one should also verify whether the physically and numerically pooled outcome is also replicated when each of the 6 ASD samples is investigated (for the dysregulated proteins), as related to their controls and whether the statistical significance still holds for those proteins when individual subject variability is calculated. The outcome of this experiment is shown in Figure 4 and Supporting Information Table 3. As observed, there is a high level of reproducibility between the outcome of the analysis per category (ASD and controls) and, as expected, the same set of proteins was dysregulated (Fig. 4 and Supporting Information Table 3), as compared with the pooled samples (Figs. 2 and 3 and Supporting Information Tables 1 and 2). When the spectral count was compared within the ASD and control categories, to identify the variability between the individual samples, we did not find dramatic individual variability for most of the proteins identified as dysregulated and statistically significant. Some of the individual values for dysregulated proteins are shown in Figure 4, which shows mean values for spectral counts and standard error of the mean. Using analysis via Student's *t* test, we indeed found a higher spectral count in ASD as compared to controls, including elevated PIP, LTF, Ig kappa chain C region, Ig gamma-1 chain C region, Ig lambda-2 chain C regions, neutrophil elastase, polymeric immunoglobulin receptor and deleted in malignant brain tumors 1 ($P<0.05$ for all except for IgG kappa chain C $P<0.005$) (Fig. 4A). Significantly higher levels of statherin ($P<0.005$) and higher levels of proline-rich phosphopeptide and histatin-1 were observed in control samples compared to ASD, approaching statistical significance ($P<0.06$). In fact, both histatin-1 and statherin were undetectable in the ASD samples (Fig. 4B).

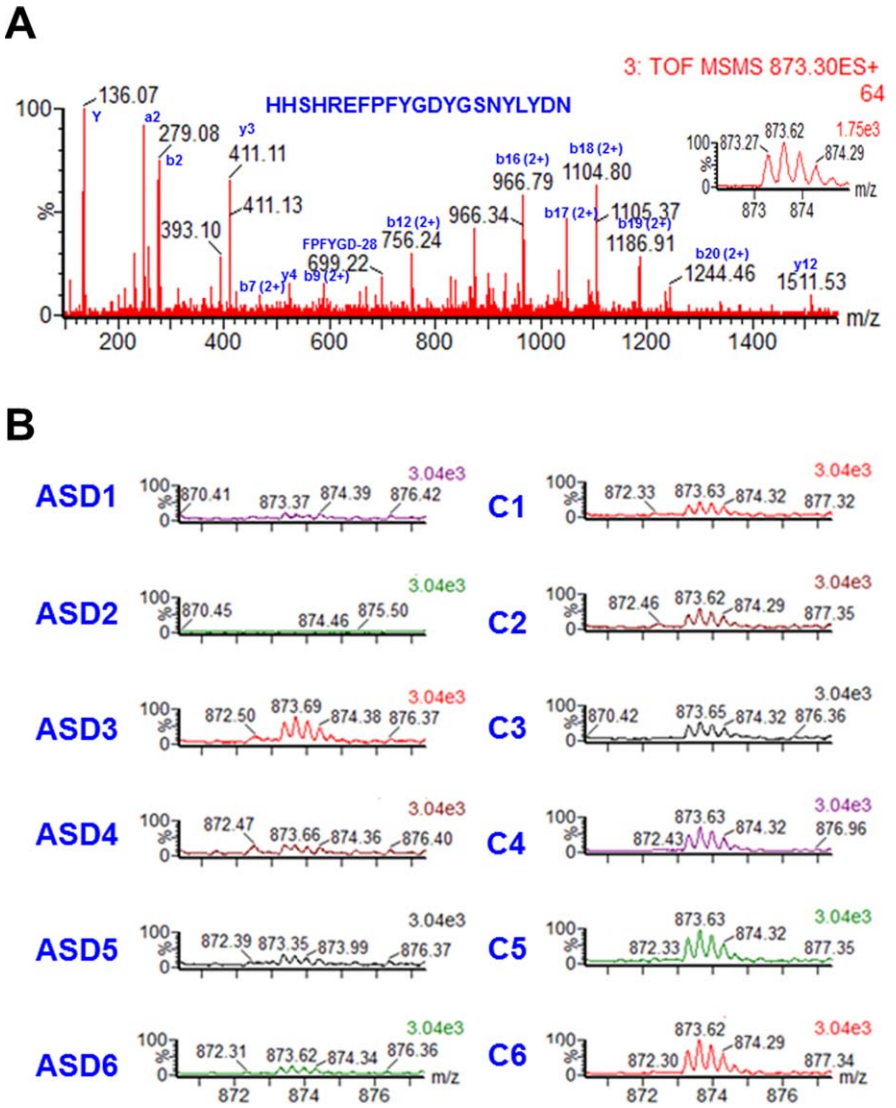

Figure 5. Relative quantitative analysis for Histatin-1 using the precursor ion intensity approach. A: The precursor ion with *m/z* of 873.30 (3+) that corresponds to peptide with the amino acid sequence HHSHREFPFYGDYGSNYLYDN, which is part of Histatin-1 was selected for fragmentation by MS/MS and produced a series of b and y ions that confirmed identification of Histatin-1. Note that the peptide is a C-terminal peptide within the amino acid sequence of Histatin-1 protein and it does not contain the traditional Arg or Lys residues as C-terminal amino acid residues (trypsin digestion usually produces peptides that have Arg or Lys as the C-terminal amino acid, unless the peptide is the C-terminal peptide, as in the case of Histatin-1 peptide). B: Comparison of the intensities of MS spectra for the precursor ion peak with *m/z* of 873.30 (3+) within the ASD and control samples. The peak with *m/z* of 873.30 (3+) has in average a lower intensity in the ASD samples, as compared with the control samples. The intensity scale for the spectra from both (−) and (+) individuals for each peptide was identical (3.04e3).

Agreement of Relative Quantitation by Spectral Counting with the Precursor Ion Intensity

To validate some of the results that we found for some dysregulated proteins in our experiments, we compared the intensity of the precursor ion over a specific period of time for a particular peptide; the precursor ion intensity was investigated in all ASD and control samples. Relative intensity for the precursor ions is a different way of performing relative quantitation, which can be used to confirm the quantitation performed by spectral count. As an example, we chose histatin-1, a protein that was decreased in the ASD samples compared with controls. We investigated a peak with the mass-to-charge ratio (*m/z*) of 873.28 (3+) that corresponded to a peptide with the amino acid sequence HHSHREFPFYGDYGSNYLYDN which is part of histatin-1. The MS/MS fragmentation for this precursor peak is shown in Figure 5. Data analysis of the MS/MS led to identification of

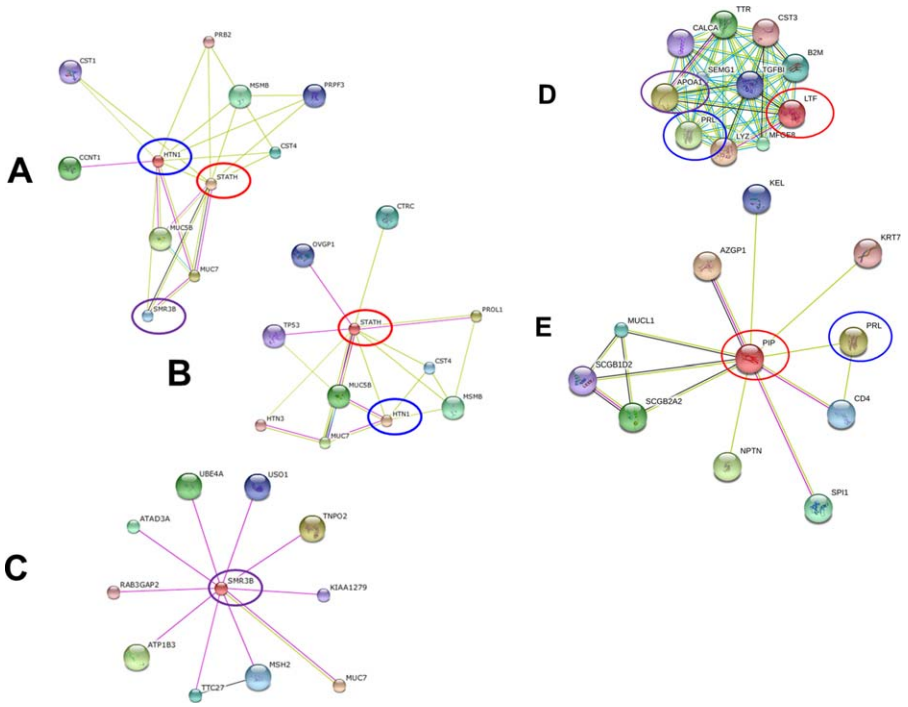

Figure 6. A–C: Investigation of the downregulated protein–protein interactions using STRING analysis. Shown are the maps with the possible interaction partners (and their predicted protein–protein interactions) for Histatin-1 or HTN1 (A), Statherin or STATH (B), and submaxillary gland androgen regulated protein 3B or SMR3B (C), as determined by STRING analysis. D, E: Investigation of the upregulated protein–protein interactions using STRING analysis. Shown are the maps with the possible interaction partners (and their predicted protein–protein interactions) for LTF or LTF (D), and Prolactin interaction protein or PIP (E), as determined by STRING analysis. Note that LTF and PIP have a common interaction partner, Prolactin (PRL). LTF is also predicted to interact with Apolipoprotein A1 or APOA1, a protein shown dysregulated in ASD in one of our previous studies.

the peptide that was part of histatin-1, with the above-mentioned amino acid sequence. The relative quantitation for the precursor ion is shown in Figure 5. A higher intensity of the precursor ion corresponds to a much abundant peptide (to make it easier, we brought all intensities in all spectra to the same intensity of 3.04e3). As observed, there is a dramatic decrease of the intensity of the precursor ion in the ASD samples, as compared with the controls. Therefore, the levels of histatin-1 are dramatically decreased in controls as compared with ASD, as determined by both spectral counting and precursor ion intensity. Similar results were also observed with statherin, a different protein that had a similar trend (decrease of the levels of this protein in ASD, as compared with controls).

Predicted Interaction of Dysregulated Proteins at the Protein Complex Level

Looking for biomarker candidates that can be used for identification of ASD (and any other disease or disorder) based on the differences in their relative abundance is just one way of looking at the dysregulated proteins. Another way of investigating biomarker candidates is through looking not only for one protein, but a protein signature, as we did in the current study. A third way of looking at biomarker candidates is through investigation of meaningful/functional post-translational modifications such as phosphorylation or acetylation. Another way of looking at biomarker candidates is based on their cellular location and biological function. A totally new way of looking at the biomarker candidates is by investigating protein–protein interactions (PPIs) between the dysregulated proteins. To investigate PPIs, we chose greatly dysregulated proteins, both upregulated and downregulated in ASD as compared with controls. For down-regulated proteins, we used submaxillary gland androgen-regulated protein 3B, histatin-1 and statherin. Histatin-1 was decreased at levels approaching statistical significance and statherin at statistically significant levels. All these proteins had decreased levels in ASD as compared with controls. As observed in Figure 6(A–C): (1) Submaxillary gland androgen-regulated protein 3B (SMR3B) is predicted to interact with histatin-1 (HTN1); (2) histatin-1 is

predicted to interact with statherin (STATH) and submaxillary gland androgen-regulated protein 3B, and (3) statherin is predicted to interact with histatin-1. Therefore, since all these proteins were downregulated in ASD and are predicted to interact with each other, this suggests that these proteins form a protein complex and it may be the intact protein complex that is downregulated in ASD. When we investigated upregulated proteins, we looked for the possible PPIs for PIP and LTF. As observed [Fig. 6(D,E)], although PIP and LTF are not predicted to interact with each other, both proteins have a common interaction partner: prolactin (PRL). Therefore, it may well be possible that these two proteins may interact with each other and are upregulated at the protein complex level. It is also worth mentioning that we identified apoA1 as a possible interaction partner, a dysregulated protein found in one of our previous studies as upregulated in ASD in sera [Ngounou Wetie, Wormwood, et al., 2014]. Taken together, these data suggest that there are dysregulation of PPIs, both higher (statherin - histatin-1) and lower (LTF—PIP) levels in ASD, compared with controls.

Discussion

We used several approaches to attempt to uncover putative ASD biomarkers in this pilot proteomic study. Initially, we screened for biomarkers using a pooled sample approach. We found significantly increased levels of several proteins. The pooled sample approach that we initially used in this study is commonly employed in proteomic studies of cancer biomarkers, and has the advantage of amplifying protein spectra, so that possible candidates may be revealed [Chen et al., 2013; Mu et al., 2013]. We initiated our study with this approach as an efficient and relatively quick method for determining whether any differences exist between the two sample groups. The detection of differences using this broad stroke approach allowed us to continue with more detailed sensitive analysis at the individual sample level. Although the pooled approach is of great utility for initial screening, this approach may mask differences in proteins at the level of the individual. This is of particular concern for the study of autism, which is a heterogeneous disorder of largely unknown cause [Talkowski et al., 2014]. We may therefore anticipate different biomarker signatures in ASD based on ASD subtypes. Therefore, we needed to move from this initial approach to analysis at the level of the individual.

Based on our individual analysis, we found statistically significant differences in several proteins, notably those known to be involved in immunological responses and inflammation. Therefore, the proteins that we found at elevated levels in ASD samples have functions that are consistent with current theories of ASD causality. For example, LTF (also called lactoferrin) is a protein involved with gastrointestinal antimicrobial activity. Thus, it is a component of immune responses of the digestive system. Gastrointestinal symptoms correspond with ASD severity and gastrointestinal symptoms are common in individuals with ASD [Adams et al., 2011]. Elevated fecal LTF has indeed been observed in some individuals with ASD [Martirosian et al., 2011], consistent with our results in saliva.

Other proteins that we identified are known components of the salivary proteome, such as prolactin inducible protein [Wu et al., 2014]. Its expression is upregulated by prolactin and androgens, and downregulated by estrogens. Increases in prolactin inducible protein are considered to be a biomarker for breast and prostate cancer [Hassan et al., 2009], therefore, it may have immune system regulatory functions. Ig gamma-1 chain C region and Ig kappa chain C region have both been associated with immunological and inflammatory diseases, particularly of the salivary glands [Donadio et al., 2013]. Neutrophil elastase is secreted as part of a response to chronic inflammation [Doring, 1994] and the polymeric immunoglobulin receptor may also be elevated as part of a mucosal immune response [Johansen & Kaetzel, 2011], and this is also true of deleted in malignant brain tumor 1 [Muller et al., 2012]. The elevated proteins that we have detected with statistically significant differences compared to control samples therefore have functions that are consistent with the concept that immune responses and inflammation may be increased in ASD [Noriega & Savelkoul, 2014; Theoharides, 2013]. It should be noted that only one subject (in the ASD group) in this study had allergies, a possible source of elevated immune responses. We did not find distinct elevations of the significantly increased proteins in that individual. Likewise, we did not find a distinct pattern in the significantly increased proteins in the individual with epilepsy and ASD or in the individual with possible Asperger's syndrome.

Interestingly, histatin-1 and salivary acidic proline-rich phosphospopeptide were elevated in control samples at levels approaching significance and statherin at statistically significant levels. In fact, histatin-1 and statherin were undetected in ASD samples. This suggests that deficits in specific salivary proteins may serve as biomarkers in ASD, comprising a signature that indicates ASD presence and risk. Indeed, hypophosphorylation of proteins such as histatin, statherin and acidic proline rich phosphopeptide has been observed in ASD, indicating that alterations in these proteins may indeed be present in ASD [Castagnola et al., 2008].

Immunological responses have been consistently observed in association with ASD, and our current study supports this idea. Epidemiological studies have

supported a relationship between ASD and a family history of autoimmune diseases. In addition, abnormal levels of various inflammatory cytokines and immunological markers have been observed in the blood of individuals with ASD in several studies [Gesundheit et al., 2013]. Finally, a recent study supports that antibodies present in some mothers of children with ASD may bind fetal brain proteins and may be an ASD risk factor [Braunschweig et al., 2013]. The present study's findings correspond with these observations and further suggest a possible mechanism for screening for ASD risk and presence.

As the subjects in this study are all male, the question of whether the conclusions of our study could be expanded to female ASD patients should be raised. We included only males in this study because only male participants were available. Research demonstrates that more males are diagnosed with ASD than females [Wingate et al., 2014]. There is however, evidence for different protein blood biomarkers in males and females with ASD, specifically Asperger's syndrome. A recent MS study reported that females had differences in proteins related to lipid transport/metabolism versus controls, whiles males with Asperger's syndrome primarily had differences in inflammatory proteins [Steeb et al., 2014], confirming a prior study showing similar differences in adult males and females with Asperger's syndrome [Schwarz et al., 2011]. Our current results support that inflammatory biomarkers are present in males with ASD in the salivary proteome, but we do not yet know if the current protein signature generalizes to females. In the only other published study of salivary proteomics in ASD, 7/27 subjects with ASD were female, however, no gender-specific differences were reported. Further studies of the ASD salivary proteome, including female subjects, would be of great interest.

In future studies, we plan to study the salivary proteome in greater detail by increasing the detection of the number of identified proteins and therefore expanding the pool of possible available biomarkers for autism. This could be done using more subjects (samples). Further work is needed to validate these findings in larger numbers of subjects with ASD and to identify whether there are specific ASD subtypes with characteristic proteomic signatures.

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