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14. ABSTRACT
Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in communication (verbal and nonverbal), social interactions, and stereotyped behaviors/interests. The etiology of ASD is not well understood, though it likely involves both genetic and environmental factors. Immune system dysfunction has been reported in ASD in many studies. Systemic immunologic alterations in autistic individuals often have been associated with autoimmunity; in particular, the generation of antibodies reactive against brain and CNS proteins. The goal of this grant is to identify serum antibody biomarkers for ASD using a novel combinatorial peptoid library that has been successful for the identification of antibody biomarkers for Alzheimer's disease. An ASD blood biomarker would be very useful for early identification and targeted therapeutic intervention. During Year-2 of the grant we have: (1) collected serum samples from additional male typically developing (TD) and ASD subjects; (2) screened libraries for peptoids using pooled serum samples from male ASD, and male TD controls; (3) Identified the sequence of the useful "male" ASD peptoids identified in Years 1 and 2; and (4) Tested the optimal peptoid for sensitivity and specificity using 51 ASD boys and 43 TD boys. In addition, we have identified two new proteins that are linked to ASD.

15. SUBJECT TERMS
ASD, autism spectrum disorders. TD, typically developing control. US, unaffected sibling control.

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Introduction: Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in communication (verbal and nonverbal), social interactions, and stereotyped behaviors/interests. The etiology of ASD is not well understood, though it likely involves both genetic and environmental factors. Immune system dysfunction has been reported in ASD subjects and in their mothers in many studies (e.g., Ashwood & Van deWater, 2004; Jyonouchi *et al*., 2005; Molloy *et al*., 2006; Braunschweig et al., 2013). Systemic immunologic alterations in autistic individuals often have been associated with autoimmunity; in particular, the generation of antibodies reactive against brain and CNS proteins. For example, both abnormalities in serum antibody concentrations and T cells have been reported for ASD compared to typically developing (TD) children (e.g., Warren *et al*., 1990; Singh, 2009). The goal of this study is to identify serum antibody biomarkers for ASD using a novel combinatorial peptoid library that has been successful for the identification of antibody biomarkers for Alzheimer's disease (Reddy *et al*., 2011). An ASD blood biomarker would be very useful for early identification and targeted therapeutic intervention.

Overall Project Summary:

For Year 2 we proposed to:

1. Screen library for IgG and IgM peptoids using pooled serum and samples 10 female ASD, 10 female TD and 10 female US controls.

We have collected a total of 73 male ASD, 18 female ASD, 66 male TD, 20 female TD, and 16 male US. To date we have focused on screening peptoid libraries for IgG-binding peptoids using the male pools. Once we find useful peptoids from screening the male ASD pools, we will determine whether they also recognize female ASD subjects. In Year-3 we will begin to search for peptoids in the IgM serum fraction.

2. Identify the sequence of the useful "male" and female" ASD peptoids identified in Years 1 and 2.

We have sequenced several "hit" ASD peptoids and focused our attention on the ASD1 peptoid. Its sequence is illustrated in **Figure 1.**

3. Test these optimal peptoids for sensitivity and specificity using 50 ASD samples, 30 TD samples and 30 US samples for each gender separately.

We have tested the ASD1 peptoid for its ability to discriminate between ASD and TD boys using 51 ASD boys and 43 TD boys. There is significantly higher binding of the ASD1 peptoid to the IgG1 fraction in the TD boys vs. ASD boys ($p<0.004$) (**Figure 2**). During Year-3 we will test the ASD1 peptoid for its ability to discriminate ASD vs. TD in the female samples.

Additional efforts

Rules Based Medicine biomarker discovery

In addition to the peptoid approach to identifying biomarkers for ASD, we have used a second approach. Using the Rules Based Medicine DiscoveryMAP 175+ luminex platform, we measured 175 serum proteins in the blood of 30 ASD boys and 30 age-matched TD boys. **Table 1** below shows the 11 proteins that were significantly different between the two groups, and also shows the "importance" of each protein as members of a panel to successfully predict ASD vs. TD after analysis with Random Forest methods. Highlighted in yellow are 6 proteins that we propose to further test with a larger sample of ASD and TD subjects *for validation purposes*, and using a different platform. We have done this with TSH, which has the highest importance for predicting ASD vs. TD. Using the Meso Scale Discovery electrochemical detection platform we found significantly lower TSH in the ASD boys vs. TD boys ($n=50$/group, $p<0.001$).

Peptoid-Antibody Identification

We have performed pull-down experiments with the ASD1 peptoid to determine the identity of the antibody binding to the ASD1 peptoid. After affinity purification of serum using the ASD1 peptoid, and observation of this analyte by Coomassie Blue staining after gel electrophoresis, we found a single band whose mass does not correspond with an antibody fragment. After sequencing this band, we identified two non-antibody proteins that were pulled down by the ASD1 peptoid: **(1) Fetuin-A**, a glycoprotein primarily involved in mineral homeostasis, has been suggested to play a neuroprotective role in the developing brain (Elsas *et al.*, 2013). In addition, mutations in Fetuin-A's corresponding gene have recently been linked to a developmental disorder (3q27.3) exhibiting some autistic features (Thevenon *et al*., 2014). We looked at the levels of Fetuin-A in some ASD and TD boys and found significantly lower levels in the ASD boys (**Figure 3**). We will measure levels of this protein in future studies to determine how it may function in a panel of biomarkers for the identification of ASD. **(2)** The other protein pulled down was **alpha-1 antitrypsin** (AAT), which has previously been linked to ASD (Russo *et al*., 2009). We wish to determine whether this protein is expressed in different levels in the ASD vs. TD children. As we were unable to pull-down an antibody in these experiments, however, the etiology of the observed IgG binding to the ASD1 peptoid has yet to be determined.

Key Research Accomplishments:

- Nearly all serum samples have been obtained and processed.
- Two unique peptoid libraries have been synthesized and validated.
- The peptoid libraries have been screened using a magnetic screening method, and peptoids were found that demonstrate a difference in IgG-binding activity between ASD, TD and US sera.
- A peptoid, ASD1, has been identified that binds significantly lower levels of IgG1 in ASD boys vs. TD boys.
- Pull-down assays were run to identify the antibody recognized by the ASD1 peptoid.
- Efforts are underway to identify the antibody that binds to the ASD1 peptoid.
- Two new proteins were identified with the ASD1 peptoid with possible links to ASD (Fetuin-A and AAT). These proteins may be useful as part of a panel of proteins that can serve as a biomarker for ASD.

Conclusions:

Several peptoids that differentiate between ASD and TD serum have been identified following screenings of a peptoid library. Contrary to expectation, however, these peptoids bind *lower* levels of IgG in ASD serum compared to TD serum. Similarly, IgG in US serum also exhibited lower binding to the peptoids, suggesting that the differential binding is related to "autism families" rather than ASD alone. The ASD1 peptoid binds significantly lower levels of IgG1 in ASD boys (n=51) compared to TD boys (n=43). Affinity-purification of serum proteins that bind to the ASD1 peptoid have revealed two proteins, Fetuin-A and AAT, that may have links to ASD.

Reportable Outcomes:

We have a poster that will be presented at the Society for Neuroscience meeting, Nov. 2014 - German, DC, Yazdani U, Singh S, Deng Y, Zaman S, Hewitson L. Blood biomarkers for Autism: peptoids and proteins. Soc. Neurosci. Abst., 2014

References:

Ashwood P, Van deWater J. (2004) Is autism an autoimmune disease? *Autoimmun Rev*, 3:557–62.

Braunschweig D, Krakowiak P, Duncanson P, Boyce R, *et al.* (2013) Autism-specific maternal autoantibodies recognize critical proteins in developing brain. *Transl Psychiatry.*3: e277.

Elsas J, Sellhaus B, Herrmann M, Kinkeldey A, Weis J, Jahnen-Dechent W, Häusler M. (2013), Fetuin-A in the developing brain. *Devel Neurobio,* 73: 354–369.

Jyonouchi H, Geng L, Ruby A, Zimmerman-Bier B. (2005) Dysregulated innate immune responses in young children with autism spectrum disorders: their relationship to gastrointestinal symptoms and dietary intervention. *Neuropsychobiol* 51:77–85.

Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, *et al.* (2006) Elevated cytokine levels in children with autism spectrum disorder. *J Neuroimmunol* 172:198–205.

Reddy MM, Wilson R, Wilson J, Connell S, Gocke A, Hynan L, German D, Kodadek T. (2011) Identification of candidate IgG antibody biomarkers for Alzheimer's disease through screening of synthetic combinatorial libraries. *Cell*, 144: 132-142.

Russo, AJ, Neville L, Wroge C. (2009) Low serum alpha-1 antitrypsin (AAT) in family members of individuals with autism correlates with PiMZ genotype. *Biomarker Insights,* 4: 45-56.

Singh VK. (2009) Phenotypic expression of autoimmune autistic disorder (AAD): A major subset of autism. *Ann Clin Psychiat.* 21:148-160.

Thevenon J, Callier P, Poquet H, Bache I, et al. (2014) 3q27.3 microdeletional syndrome: a recognizable clinical entity associating dysmorphic features, marfanoid habitus, intellectual disability and psychosis with mood disorder. *J. Med. Genet.,* 51:21-27.

Warren RP, Yonk LJ, Burger RA, Cole P, *et al*. (1990) Deficiency of suppressor-inducer ($CD4^+CD45RA^+$) T cells in autism. *Immunol Invest* 19:245–51.

Appendices:

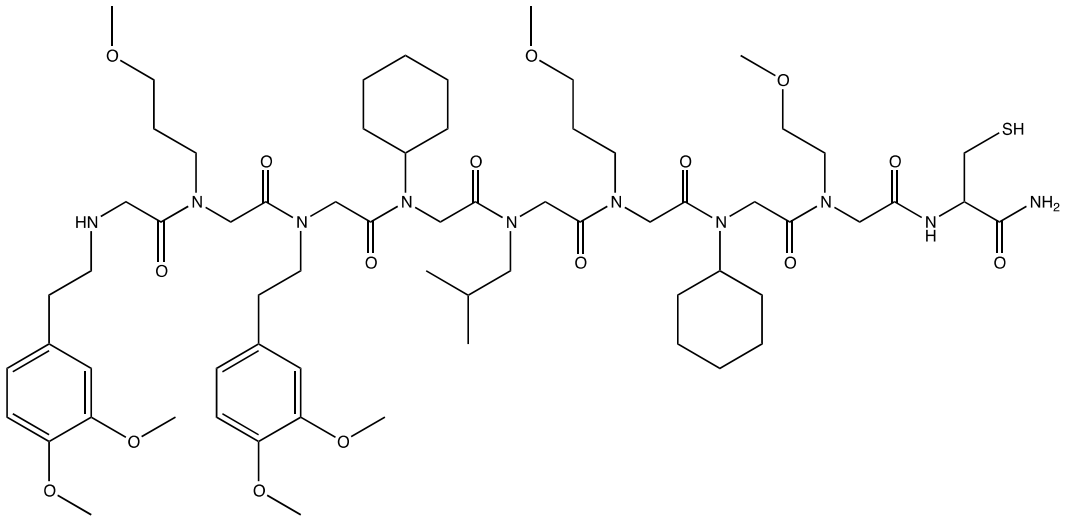

Figure 1. Sequence of the ASD1 peptoid. This sequence corresponds to: N - Ndmpa - Nmpa - Ndmpa - Ncha - Nleu - Nmpa - Ncha - Nmea - Cys.

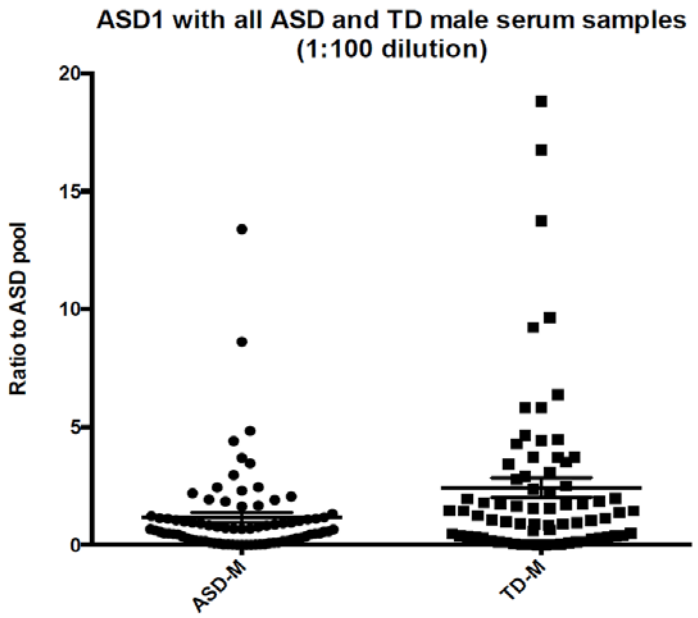

Figure 2. The ASD1 peptoid binds lower levels of IgG1 in ASD boys. We used 51 ASD and 43 TD samples ($p<0.004$). Error bars show SEM.

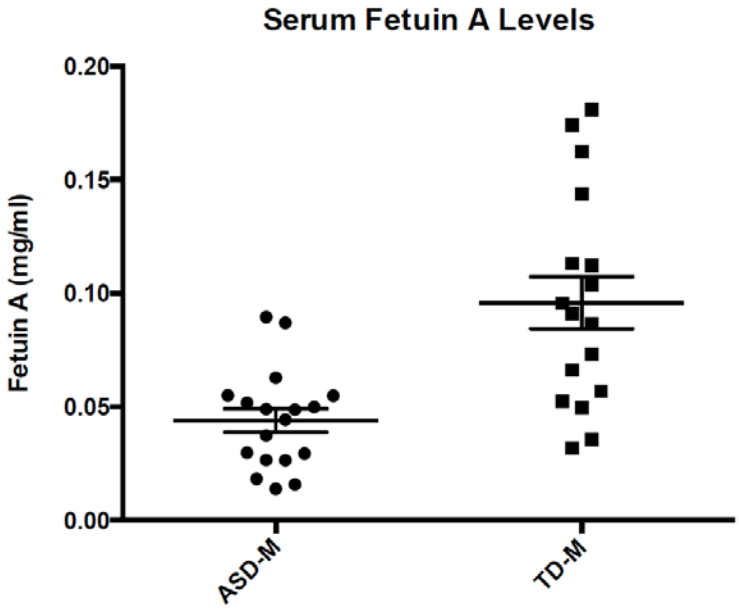

Figure 3. Fetuin-A levels are significantly lower in ASD boys vs. TD boys. We compared 18 ASD and 17 TD subjects ($p<0.001$). Error bars show SEM.

Table 1. Proteins that differ in ASD vs. TD serum samples (n=30/group).

	Protein	t-test	Importance
1	Alpha.1.Microglobulin..A1Micro.	0.01735876	2.30937619
2	Apolipoprotein.E..Apo.E.	0.0357052	-1.865839683
3	Apolipoprotein.H..Apo.H.	0.10348344	4.153229365
4	AXL.Receptor.Tyrosine.Kinase..AXL.	0.05962279	-0.233159524
5	Chromogranin.A..CgA.	0.05041011	-0.418786508
6	Ferritin..FRTN.	0.05617234	3.295528788
7	Interleukin.8..IL.8.	0.04323411	2.568924603
8	Monocyte.Chemotactic.Protein.4..MCP.4.	0.06424976	3.393397475
9	Monokine.Induced.by.Gamma.Interferon..MIG.	0.16632776	2.088083333
10	Stem.Cell.Factor..SCF.	0.00802738	4.356692785
11	Thyroid.Stimulating.Hormone..TSH.	0.00356739	14.63983175