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PRINCIPAL INVESTIGATOR: Dwight German, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Southwestern Medical Center, Dallas TX 75390

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Dwight German

E-Mail: dwight.german@utsouthwestern.edu

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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 proposal 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-1 of the grant we have (1) collected serum samples from additional male typically developing (TD) and unaffected sibling (US) controls; (2) collected serum samples from additional female TD and US controls; (3) made peptoid libraries; and (4) screened libraries for peptoids using pooled serum samples from male ASD, and male TD controls.

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.

Body:

For Year 1 we proposed to:

1) Collect serum samples from additional male TD and unaffected sibling (US) controls.

We have on hand a total of 30 TD male serum samples and 20 US male serum samples. We will collect the remaining 10 US male samples during Year 2.

2) Collect serum samples from additional female TD and US controls.

We have on hand a total of 30 TD female serum samples and 18 US female serum samples. We will collect the additional female US samples during Year 2.

3) Make peptoid library (>2 million peptoids).

Two distinct one-bead one-compound peptoid libraries of approximately 6 million beads each (TentaGel, 75μm) have been successfully synthesized. The first library was configured as NH_2-X_7-Nmea-Nmea-Met-TentaGel, where X = Nall, Nasp, Ncha, Nffa, Nleu, Nmba, Nmpa, Nphe, Npip, Npyr, Nser, yielding a theoretical diversity of 11^7 possible compounds (see **Figure 1**). This library was used for the first round of screens using pooled serum samples from 10 ASD and 10 TD males (age 2-8 years/group).

A second library was then created with less hydrophobic character in an attempt to decrease the large number of false positive "hits" yielded during the first round of screens. This library was configured as NH_2-X_6-Nmpa-**Nlys**-Met-TentaGel, where X = Nall, Nasp, Ncha, Nippa, Nleu, Nlys, Nmba, Npip, Npyr, Nser, yielding a theoretical diversity of 10^6 possible compounds. Although the theoretical diversity is lower for this library, the amine submonomers used carry a broader chemical functionality than those used in the first library. In addition, the charged Nlys residue included in the linker decreases the hydrophobic character of the library as a whole.

In each, proper library synthesis was confirmed by CNBr cleavage of compounds from a sample of isolated beads and subsequent analysis by tandem mass spectrometry.

4) Screen library for IgG and IgM peptoids using pooled serum samples from 10 male ASD, 10 male TD and 10 male US controls.

Multiple screens for ASD-specific IgG-binding peptoids have been performed using the first library mentioned above. Serum pools were made using 10 male TD samples and 10 male ASD samples. These pools were then used in a screening method using magnetized secondary antibodies to isolate "hit" beads containing peptoid bound to IgG from the pools. The TD pool was used to deplete the library of peptoids binding normally-present IgGs, and the ASD pool was then used to isolate "hit" peptoids.

From these magnetic screens, 402 "hit" beads were isolated. The peptoids from 25 of these "hits" were sequenced and 7 were subsequently synthesized and validated on an ELISA platform. Surprisingly, all seven of the peptoids demonstrated an unexpected pattern of binding in which IgG from the TD serum pool appeared to bind to the peptoids with higher amounts than IgG from the ASD serum pool (**Figure 2a**). We then individually tested the 10 ASD and 10 TD serum samples that constitute each pool against one of the peptoids, called ASD1.

Consistent with the results of the pooled serum, the individual serum samples tended to bind lower in the ASD group than in the TD group (**Figure 2b**). An ELISA was then run using the ASD1 peptoid against *IgG purified* from the two serum pools. In contrast with the pooled serum, the purified IgG from the ASD and TD pools showed no difference in binding to the peptoid and further exhibited similar high-affinity binding (**Figure 2c**). These data together suggest that a non-IgG factor that interferes with the binding of IgG to the peptoid exists at higher levels in the serum of the ASD subjects versus the TD subjects.

To further test the specificity of the ASD1 peptoid we pooled 10 male US serum samples and compared it with the ASD and TD pools. **Figure 3** shows that the binding of IgG in both the ASD and US serum pools is markedly lower than that of the TD pool. These data suggest that the serum IgG binding patterns observed for the ASD1 peptoid are not specifically related to ASD per se, but rather to "autism families". Other studies have also found similarities in immune molecules in ASD and US children vs. TD children (Saresella *et al*., 2009; Napolioni *et al.,* 2013).

As mentioned above, a second library with less hydrophobic character was synthesized with the intent to reduce the large number of "hits" to hopefully increase the specificity of the screens. Magnetic screens of this second library are ongoing. We hope to find peptoids that demonstrate specificity to ASD by demonstrating differential binding between the ASD and US groups.

Additional efforts

In addition to our proposed goals, we performed a number of other analyses relevant to the study. A study by Heuer *et al*. (2008) demonstrated slightly reduced levels of IgG in children with ASD versus age-matched TD children. Further, they observed a slight increase in the level of the IgG4 subclass in children with ASD versus age-matched TD children. We performed quantitative analyses of both total IgG and IgG subclasses on our ASD and TD samples, from both males and females, to see if these differences were reflected in our samples. In all cases, we observed no significant differences between groups in either total IgG or any of the IgG subclasses (e.g. **Figure 4a**). As expected, however, the levels of IgG1 were observed to be the highest among the four IgG subtypes in all samples. Using a secondary antibody specific to IgG1, we repeated the ELISA experiments mentioned above. While the IgG1 subtype demonstrated a similar pattern of binding to the ASD1 peptoid among the ASD, TD and US groups (**Figure 4b**), the level of binding of the ASD and US groups was decreased (as compared with total IgG in Figure 3), thereby increasing the magnitude of difference between the TD and ASD groups. This increase in magnitude was also observed when testing the individual serum samples from the three groups using the IgG1-specific secondary antibody (**Figure 4c**). Since the IgG1-specific secondary antibody recognizes a more precise epitope than the IgG-specific secondary antibody used in the earlier ELISAs, it can be interpreted that the increased magnitude observed in these data is the result of a decrease in "background noise". In future studies we will additionally use the IgG1 subtype to screen for and evaluate "hit" peptoids.

Due to the qualitative nature of the magnetic screening method ("hit" beads are assessed simply by being sufficiently "magnetized" to be pulled out of the peptoid library pool) we have been seeking a more quantitative high-throughput method so that we may assess the binding strength of our "hit" peptoids prior to the laborious process of sequencing, re-synthesizing, and validating on ELISA. To this end, we have been exploring fluorophore-tagged secondary antibodies and subsequent separation of "hits" from "non-hits" using a cell sorter. Efforts are ongoing to make this method practicable.

Lastly, Gao and Kodadek (2013) have recently demonstrated that a slight modification to the basic peptoid structure can yield more "rigid" molecules that bind antibody with significantly higher affinity. These modified peptoids can be referred to as peptide tertiary amides (PTAs). We have demonstrated the ability to synthesize these PTAs in our own lab, and are currently seeking to create a PTA library to use for screening.

Key Research Accomplishments:

- Nearly all serum samples have been obtained and processed.
- Two unique peptoid libraries of 6 million beads each have been synthesized and validated.
- A peptoid library has 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.

- No significant difference was observed in total IgG and IgG subclass levels between ASD and TD groups, male or female.
- Efforts are underway to improve current screening methods and enhance library quality.

Reportable Outcomes:

We have published a review paper on the neuropathology of autism and animal models of the disease - Gadad, B.S., Hewitson, L., Young, K.A., German, D.C. Neuropathology and animal models of autism: genetic and environmental factors. *Autism Research and Treatment,* vol. 2013, Article ID 731935, 12 pages, 2013. doi:10.1155/2013/731935.

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. Further investigation into the etiology of this differential binding may reveal specific differences between ASD and TD sera. Continued screens using improved libraries and screening methods will be undertaken to yield compounds suitable for antibody biomarker identification in ASD.

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Appendices:

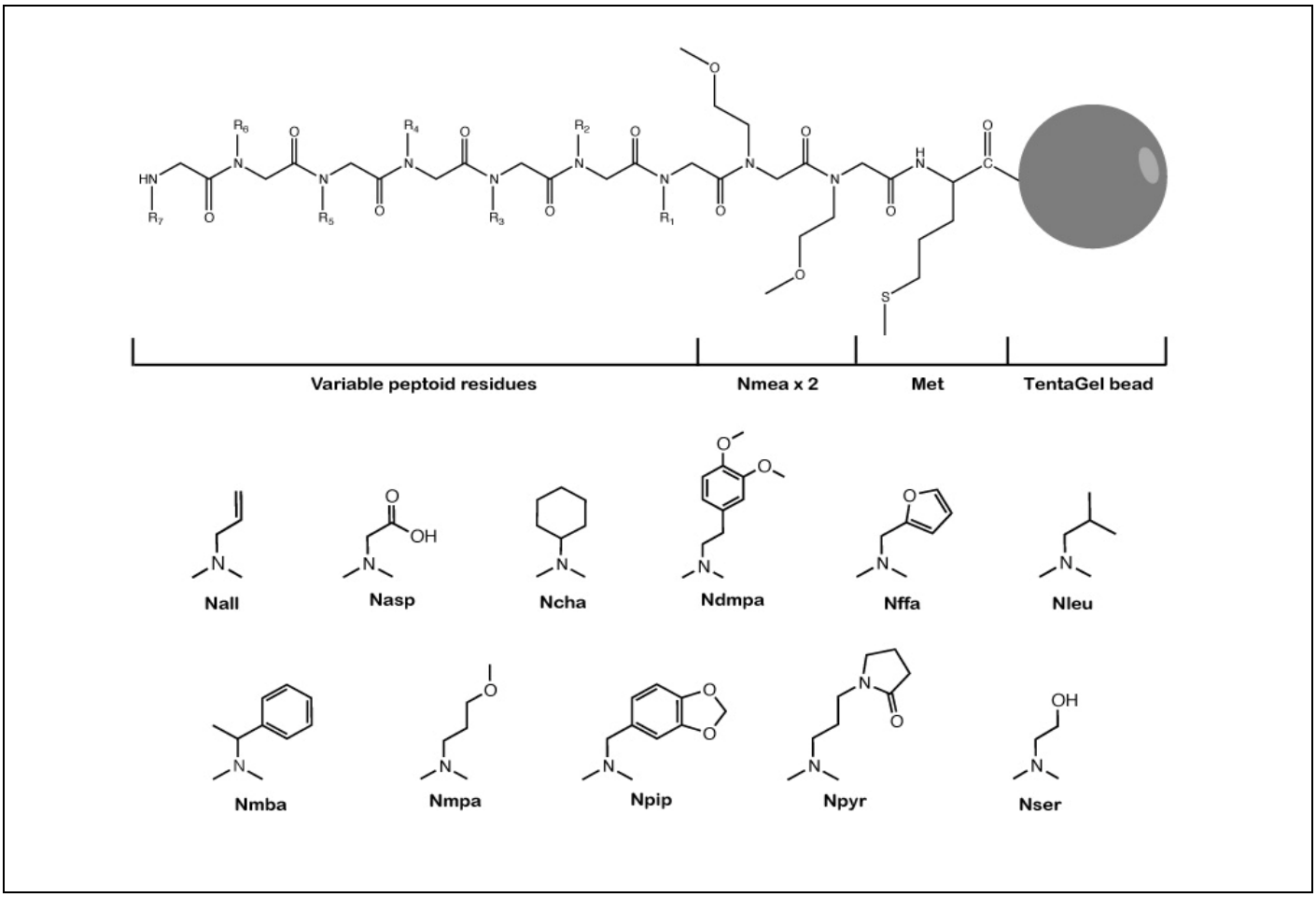
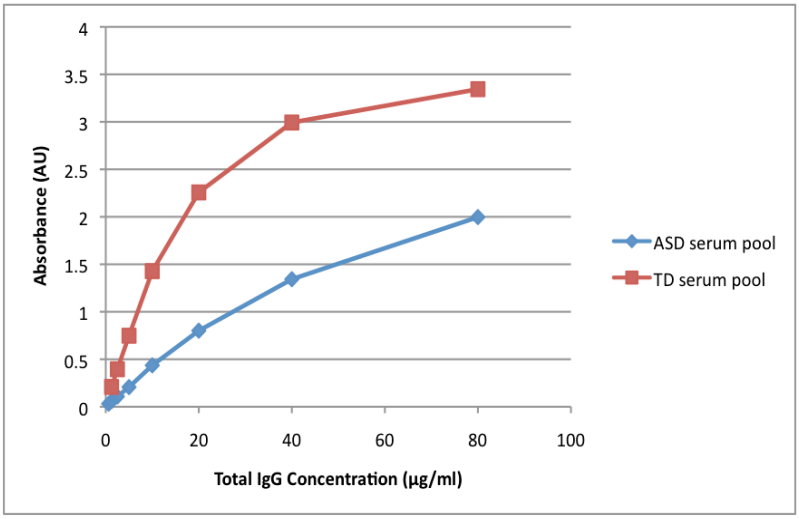


Figure 1. Structure of the first peptoid library. The structures of the 11 amine submonomers used are shown at the bottom. For example, Nser - ethanolamine, Npip – piperonylamine, Nasp - glycine, Nmba – (R)-methylbenzylamine, Nleu – isobutylamine, Nall – alylamine, Nlys – diaminobutane.

A.



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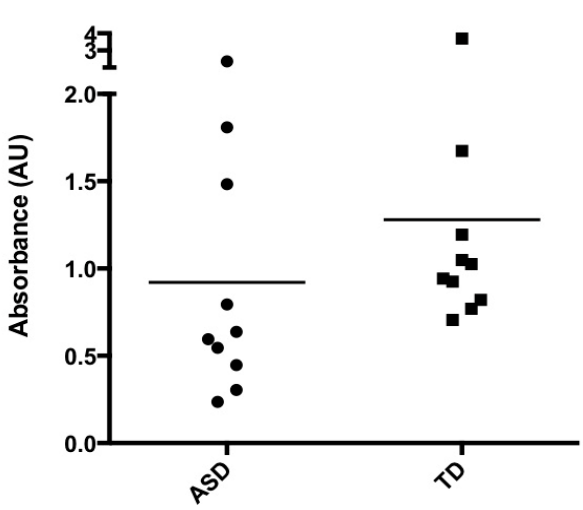

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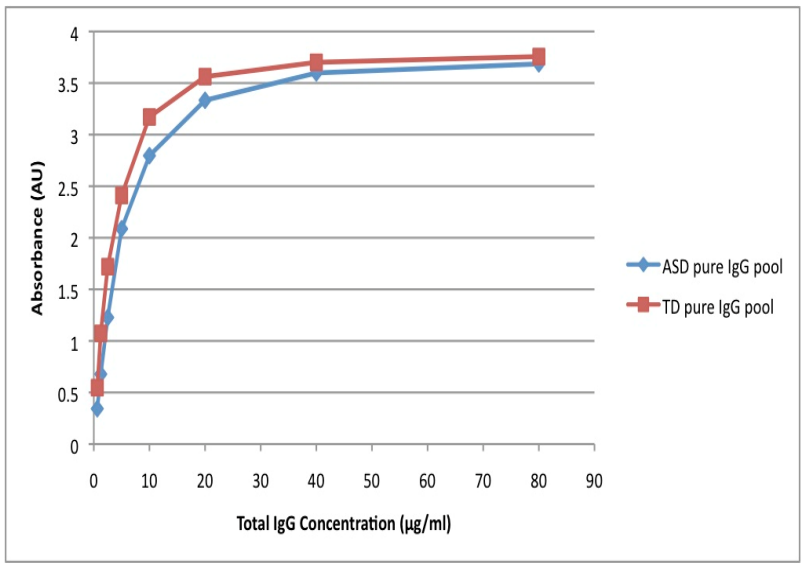

Figure 2. ELISAs for the ASD1 peptoid. (A) The ASD1 peptoid recognizes higher levels of IgG in the pooled TD serum versus the pooled ASD serum. **(B)** Likewise, the 10 individual serum samples constituting the ASD pool (1:400 dilution) tended to show lower IgG binding to the ASD1 peptoid compared to the 10 individual serum samples of the TD pool. **(C)** When the ASD1 peptoid was tested against purified IgG from the two pools, however, no difference was observed, and the peptoid showed similar high affinity binding to the IgG in both pools. This suggests that the ASD subjects have a non-IgG serum factor that interferes with the binding of IgG to the ASD1 peptoid.

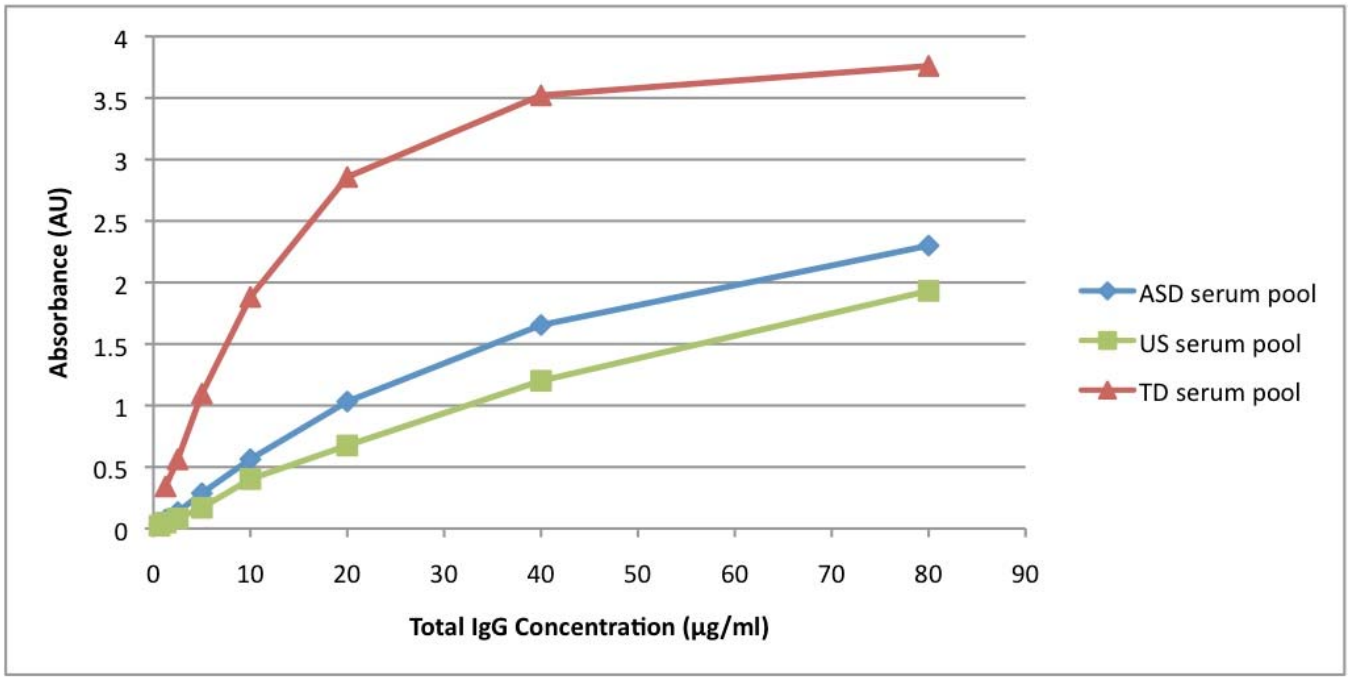

Figure 3. ASD1 binding to IgG in serum from ASD, TD and US pools. The ASD1 peptoid binds high levels of antibody in the TD serum, and much lower levels in the ASD serum. The IgG binding is similarly low for the US serum pool suggesting that the differential binding to ASD1 is related to "autism families".

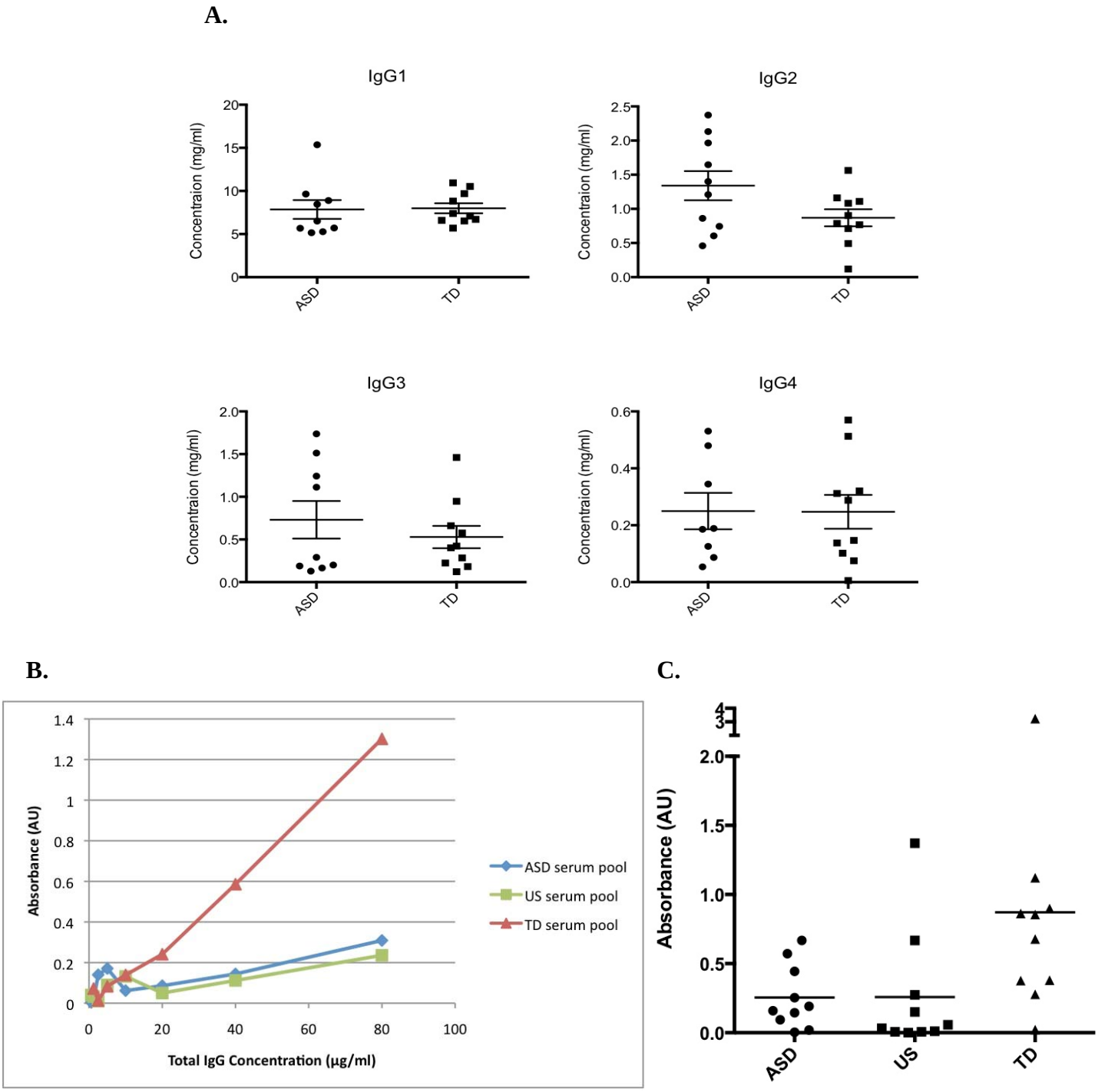

Figure 4. IgG subtypes in ASD and TD serum samples. (A) No significant difference was observed between male ASD and TD serum samples for amounts of IgG1-4. Similar results were observed for the female ASD and TD samples (data not shown). As expected, IgG1 is the most abundant of the subtypes. **(B)** The ASD1 peptoid also recognizes higher levels of the IgG1 subtype in the TD serum pool versus the ASD and US pools. Note that the magnitude of difference between the TD and ASD pools at 80µg/ml is larger than that of the same concentration in Figure 2a. **(C)** Individual ASD, US and TD samples (n=10/group, 1:100 dilution) indicate lower IgG1 binding to the ASD1 peptoid in the ASD and US subjects compared to the TD subjects, similar to the results illustrated with the pooled samples in 4B.