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## Peptides of neuron specific enolase as potential ASD biomarkers: From discovery to epitope mapping

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### Abstract

Autism spectrum disorder (ASD) is an important health issue and affects 1 in 59 children in the US. Prior studies determined that maternal autoantibody related (MAR) autism is thought to be associated with ~23% of ASD cases. We previously identified seven MAR-specific autoantigens including CRMP1, CRMP2, GDA, LDHA, LDHB, STIP1, and YBX1. We subsequently described the epitope peptide sequences recognized by maternal autoantibodies for each of the seven ASD-specific autoantigens. The aim of the current study was to expand upon our previous work and identify additional antigens recognized by the ASD-specific maternal autoantibodies, as well as to map the unique ASD-specific epitopes using microarray technology. Fetal Rhesus macaque brain tissues were separated by molecular weight and a fraction containing bands between 37 and 45 kDa was analyzed using 2-D gel electrophoresis, followed by peptide mass mapping using MALDI-TOF MS and TOF/TOF tandem MS/MS. Using this methodology, Neuron specific enolase (NSE) was identified as a target autoantigen and selected for epitope mapping. The full NSE sequence was translated into 15-mer peptides with an overlap of 14 amino acids onto microarray slides and probed with maternal plasma from mothers with an ASD child and from mothers with a Typically Developing child (TD) (ASD = 27 and TD = 21). The resulting data were analyzed by T-test. We found 16 ASD-specific NSE-peptide sequences for which four sequences were statistically significant ( $p < 0.05$ ) using both the  $t$ -test and SAM  $t$ -test: DVAASEFYRDGKYDL ( $p = 0.047$ ; SAM score 1.49), IEDPFDQDDWAAWSK ( $p = 0.049$ ; SAM score 1.49), ERLAKYNQLMRIIEEE ( $p = 0.045$ ; SAM score 1.57), and RLAKYNQLMRIIEEL ( $p = 0.017$ ; SAM score 1.82). We further identified 5 sequences that were recognized by both ASD and TD antibodies suggesting a large immunodominant epitope (DYPVVSIEDPFDQDDWAAW). While maternal autoantibodies against the NSE protein are present both in mothers with ASD and mothers of TD children, there are several ASD-specific epitopes that can potentially be used as

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#### Declaration of Competing Interest

The authors (JV, EE and ARC) have a patent application involving the peptides described in this research paper. The other authors have no conflicts of interest.

#### Appendix A.: Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2019.12.002>.

MAR ASD biomarkers. Further, studies including analysis of NSE as a target protein in combination with the previously identified MAR ASD autoantigens are currently underway.

## Keywords

Autism; Autoantibody; Epitope; Mimitope; Microarray; Peptide; Protein; 2-D gel; Western blot

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## 1. Introduction

Autism spectrum disorder (ASD) is a complex set of behavioral disorders that are characterized by two main features: 1) impairments in verbal and non-verbal communication skills with deficits in social interaction, as well as 2) the presence of restricted interests and repetitive behaviors. It is estimated that 1 in 59 children in the United States are affected by ASD (Baio et al., 2018). While the etiologies of ASD are not fully understood, studies suggest that a combination of genetic predisposition, exposure to environmental insults during gestation and/or shortly after birth, and sex of the child are important factors associated with the disease manifestations (al-Haddad et al., 2019; Bai et al., 2019; Hallmayer et al., 2011; Kim and Leventhal, 2015).

In recent years, it has been shown by several groups that maternal autoantibodies reactive to specific combinations of proteins expressed in fetal brain can impact the neurodevelopment of the offspring. This subtype of ASD has been termed Maternal Autoantibody Related (MAR) Autism, and has been observed in several clinical populations with a high degree of specificity for ASD (Braunschweig et al., 2008; Braunschweig et al., 2012; Braunschweig et al., 2013). Further, multiple murine (Braunschweig et al., 2012; Camacho et al., 2014; Jones et al., 2018; Singer et al., 2009) and non-human primate (Bauman et al., 2013; Martin et al., 2008) models have demonstrated that a strong correlation exists between the presence of these ASD-specific maternal autoantibodies with altered neurodevelopment and behavioral deficits in the progeny.

We first described the presence of maternal antibody reactivity to fetal brain proteins at 37, 39 and 73 kDa in mothers with ASD children (Braunschweig et al., 2008, 2012) and later identified these antigens as collapsin response mediator proteins 1 and 2 (CRMP1, CRMP2 ~ 73 kDa), guanine deaminase (GDA ~ 49 kDa), lactate dehydrogenase A and B (LDHA, LDHB ~ 37 kDa), stress induced phosphoprotein-1 (STIP1 ~ 73 kDa) and Y-box binding protein 1 (YBX1 ~ 39 kDa) (Braunschweig et al., 2013). In addition, our laboratory performed epitope mapping of the original seven antigens and determined specific epitopes for each protein that differentially separated the ASD and TD groups (Edmiston et al., 2017). In the current study, we built upon our first antigenic determination work to determine which samples were reactive to bands near 37–39 kDa, but unreactive to LDHA and B, YBX-1, or GDA and could be used to identify additional targets using a 2-D gel electrophoresis and proteomic analytic approach. This methodology led to the identification of neuron specific enolase (NSE) as a potential eighth antigen in the MAR ASD autoantibody repertoire.

NSE is a catalytic enzyme expressed on neurons and neuroendocrine tissues that mediates the conversion of 2-phosphoglycerate (2PG) to 2-phosphoenol pyruvate (2PEP) and the reverse reaction (2PEP to 2PG) in the glycolysis and gluconeogenesis pathways, respectively (Fukano and Kimura, 2014). For eukaryotic cells, there are three enolase isoforms that are encoded by different genes and with tissue-specific expression;  $\alpha$  enolase (ENO 1) is ubiquitously expressed,  $\gamma$  enolase (ENO 2) is found exclusively in neurons, and  $\beta$  enolase (ENO 3) is found only in muscle. The enolases are present as dimers and their function depends on the natural cofactor Mg<sup>+</sup> to regulate the conformational and catalytic activity of the enzyme (Isgro et al., 2015). In the brain, NSE is expressed as  $\gamma\gamma$  on neurons and  $\alpha\gamma$  on microglia, astrocytes and oligodendrocytes. Non-neural enolase (NNE,  $\alpha\alpha$  dimer) is observed on neural tissue during the early phase of development, but changes to the  $\gamma\gamma$  and  $\alpha\gamma$  isoforms (NSE) as neural and glia differentiation and maturation take place, and it has been implicated in cell metabolism, modulation of the immune response, neuroinflammation, neurodevelopment, and brain homeostasis by regulating cell survival/death signals (Haque et al., 2018). Thus, the potential for NSE as a target for maternal autoantibodies in the context of ASD is well-founded due to its clear role in neurodevelopmental biology.

In the current study, we describe our approach to the identification of NSE as an additional autoantibody target for MAR ASD. Furthermore, based on our previous epitope mapping studies, it was of interest to better understand the value of this antigenic target in the context of its antigenic epitopes. Therefore, in the present study, we describe the significance of ASD-specific epitopes for NSE that relate to behavioral outcome and help define maternal risk for having a child with ASD.

## 2. Materials and methods

### 2.1. Study subjects

This study included mothers enrolled in the CHARGE study (Childhood Autism Risks from Genetics and Environment) at the MIND Institute at UC Davis (Hertz-Picciotto et al., 2006). The CHARGE study participants in this study included mothers with children diagnosed with ASD (n = 246) and with children selected from the general population (typically developing, TD; n = 149). We used the recruitment, eligibility, and psychometric assessment procedures as previously described (Braunschweig et al., 2012; Hertz-Picciotto et al., 2006). ASD diagnosis was verified at the MIND Institute according to the Diagnostic and Statistical Manual of Mental Disorders–5 (DSM-5) (Association, 2013). All the procedures were approved by the California Committee for the Protection of Human Subjects and institutional review boards at UC Davis and UC Los Angeles. Prior to participation, subjects provided written informed consent in either English or Spanish.

### 2.2. Sample collection and preparation

Blood was collected in citrate dextrose (BD Diagnostic) and plasma was separated, coded, aliquoted, and stored at  $-80^{\circ}\text{C}$ . Prior to use, samples were thawed and centrifuged at 13,000 RPM for 10 min.

### 2.3. Fetal brain antigen preparation

Tissue processing was done as previously described (Braunschweig et al., 2013). Briefly, we used embryonic 152 day-old fetal rhesus macaque brain (FMB) that was supplied by the California National Primate Research Center. The FMB was mechanically homogenized with buffer using a Polytron 3000 homogenizer (Brinkman), sonicated for 3 min, and centrifuged for 10 min at  $3000 \times g$ . The supernatant was then collected, concentrated via ultrafiltration, and measured for its protein content via bicinchoninic acid assay (BCA).

### 2.4. Prep cell

Protein fractionation was performed as described previously (Braunschweig et al., 2013). Briefly, 40 mg of FMB was electrophoresed and separated by molecular weight using a Prep Cell apparatus (Bio Rad, Hercules, CA) on a 10% poly-acrylamide gel for 17 h at 12 W. Protein fractions were collected at 5-minute intervals at a flow rate of 0.75 ml/min. A total of 110 fractions were obtained, concentrated to 5 mg/ml by ultrafiltration, and probed by western blot (WB) to determine molecular weight and antigen reactivity (Fig. 1). Ponceau staining confirmed protein enrichment and fractions with a range of approximately 5 kDa per/fraction. Fraction #12 contained proteins between 37 and 45 kDa and was therefore selected to use for antigen identification (Fig. 2).

### 2.5. Western blot

To test autoantibody reactivity to FMB Fraction #12 that contained proteins between 37 and 45 kDa, the fraction was probed with maternal plasma samples as described previously (Fig. 1D) (Braunschweig et al., 2013). In summary, 200  $\mu$ g of protein were denatured by heating at 100°C for 10 min in SDS buffer and separated on a 12% SDS-PAGE gel at 200 V for 1 h. Proteins were transferred to a 0.2  $\mu$ m nitrocellulose membrane overnight (10 V for 16 h) at 4 °C. To confirm the transfer, the membrane was stained with Ponceau dye and cut into 3 mm strips that were labeled and blocked with 1% casein buffer. Plasma samples were then diluted (1:400), added to the strips, incubated for 1.5 h at RT followed by five washes, and incubated with 1:20,000 goat anti-human IgG-HRP for 30 min. After five washes, detection was performed by adding 800  $\mu$ l of Super Signal substrate and strips were placed on a glass plate to be imaged using the FluoroChem 8900 imager. Images were scored as 0 if negative and 1 if positive.

### 2.6. Two-dimensional (2-D) gel electrophoresis

Protein fractions that were targeted by maternal autoantibodies were separated by 2-D electrophoresis as described previously (Braunschweig et al., 2013). Briefly, 300  $\mu$ g of the protein fraction in the 30–40 kDa range were labeled with Cy2 (GE Life Sciences, Pittsburgh, PA, USA) in preparation for 2-D electrophoresis (all gels were done in duplicate). First, 15  $\mu$ g of each sample was separated by its isoelectric point by using 3–10 isoelectric focusing strips (GE Healthcare, Piscataway, NJ, USA). The strips were then loaded onto 2 10.5% polyacrylamide gels (GE Healthcare) for second dimension electrophoresis. Images were captured using Quant software (version 6.0, GE Healthcare). One of the gels was transferred to nitrocellulose membrane to assay for maternal plasma reactivity to bands near 37–39 kDa, but unreactive to, GDA, LDHA/B, and YBX1 by WB.

The resulting positive spots were mapped back to the Cy2 stained duplicate 2-D gel, picked from the gel, and digested with trypsin (Promega, Madison, WI, USA) in preparation for Mass spectrometry analysis (Fig. 2).

## 2.7. Mass spectrometry

Mass spectrometric analysis was performed as described in our previous report (Braunschweig et al., 2013). The digested peptides were desalted (Zip-tip C18, Millipore, Billerica, MA, USA) and spotted on the MALDI plate (model ABI 01-192-6-AB). The ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA) was used to obtain MALDI-TOF MS and TOF/TOF tandem MS/MS data. The obtained peptide mass and the associated fragmentation spectra were analyzed using a GPS Explorer workstation equipped with MASCOT search engine (Matrix Science, Boston, MA, USA) and used to perform a BLAST search on the NCBI. Candidates with either protein score confidence interval (C.I. %) or Ion C.I.% of greater than 95 were considered positive (Supplementary Table 1).

The top 4 commercially available antigens identified by mass spectrometry with a 100C.I. were selected for further evaluation. To evaluate antibody reactivity against our top hits including NSE, NNE, ALDOC, and CKB, 2 µg protein of recombinant protein (Novus Biologicals, Littleton, CO) were probed with diluted maternal plasma (1:800) by WB as described previously.

## 2.8. Enzyme linked immunosorbent assay (ELISA)

Once NSE was identified as a viable antigenic candidate by WB, we evaluated a larger sample set for NSE reactivity using an ELISA method. We tested plasma from 418 mothers enrolled in the CHARGE study with at least one child with ASD (n = 232) or control samples from mothers of typically developing children (TD; n = 186). Microtiter plates were coated with 100 µl of NSE (Novus Biologicals, Littleton, CO) at 2 µg/ml in carbonate coating buffer pH 9.6, incubated overnight at 4 °C, washed four times with PBST 0.05%, and blocked with 2% Super Block (Thermo Scientific, Rockford, IL) for 1 h at room temperature (RT). The plasma samples were diluted 1:500 and run in duplicate. Following dilution, 100 µl of diluted sample was added to each well, incubated for 1.5 h, washed 4X, then incubated with 1:10,000 goat anti-human IgG-HRP IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MA) for 1 h. The plates were then washed and detection was performed by adding 100 µl of BD optEIA liquid substrate for ELISA (BD Biosciences, San Jose, CA). After 4 min, the reaction was stopped with 50 µl of 2 N HCl. The absorbance was measured at 490–450 nm using an iMark Microplate Absorbance Reader (Biorad, Hercules, CA, USA).

## 2.9. Receiver operating characteristic (ROC) curve

For the ELISA assay, positive cutoff values for reactivity to NSE were determined using a ROC curve. The ROC curve was created by plotting the *true positive rate* against the *false positive rate* at various threshold settings. We therefore created our curve using seven positive samples (labeled as +) from mothers that have a child with ASD and were positive by WB (true positive samples) along with the test samples. By using the positive samples as the reference event, the cutoff has greater specificity (less false positives) although

sacrificing some sensitivity (limit of detection). The ROC plots sensitivity versus 1-Specificity for each value creating an Area Under the Curve (AUC) that is a representation of the accuracy of the test. Youden's index was used to calculate the cutoff (Fluss et al., 2005; Hajian-Tilaki, 2013).

## 2.10. Microarray screening

The full NSE sequence (NP\_001966.1) was obtained from NCBI and translated into a library of contiguous 15-mer peptides with a peptide-peptide overlap of 14 amino acids (aa) onto microarray slides. The discovery peptide microarrays were synthesized by PEPperPRINT as previously described (Schirwitz et al., 2012) whereby the targeted 15-mer peptide sequences are directly printed onto a glass slide in duplicate using solid-phase Fmoc chemistry (PEPperPRINT, Heidelberg, Germany). Peptides derived from human influenza hemagglutinin (HA) (YPYDVDPYAG) and the Polio vaccine (KEVPALTAVETGAT) were also included as positive controls.

To test for antibody reactivity against the printed peptides, we probed the arrays with plasma from mothers enrolled in the CHARGE study (ASD = 27 and TD = 22) according to the manufacturer's instructions. The demographic information related to these samples is shown in Table 1. The microarray slides were first incubated with standard buffer (PBS containing 0.05% Tween 20, pH 7.4) for 10 min and then blocked for 45 min at RT (Rockland Blocking Buffer MB-070; Rockland Immunochemicals Inc). The slides were then incubated overnight shaking at 4 °C with individual maternal plasma samples diluted 1:250 in staining buffer followed by 3 washes in standard buffer. For signal detection, the slides were incubated for 30 min at RT with goat anti-human IG (H + L)-DyLight649 (Rockland Immunochemicals Inc.) at a dilution of 1:5000 in staining buffer (standard buffer with 10% blocking buffer). Following secondary antibody incubation, the microarrays were imaged using the GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, California).

Fluorescence signal quantification of spot intensities (FI) and peptide annotation was done using PepSlide Analyser software (PEPperPRINT) based on manufacturer's recommendations. The data pre-processing methodology was performed as reported in previous peptide microarray studies. Briefly, net fluorescence intensities (FI) were calculated using the correction method reported by Zue et al (Zhu et al., 2006; Duarte et al., 2013). A 3X2 window was set for each spot and the median of the six spots was used as the "neighborhood background" for the central spot. In order to normalize the net Fluorescence intensities (FI) a 3X1 "slide window" was set to each spot, and the median of the three was used as the normalized signal for the central spot (Zhu et al., 2006; Duarte et al., 2013). The corrected net intensity was calculated by subtracting the corrected background from the normalized signal. If the background signal was higher in the background compared to the spot (negative FI), the signal was set to 1 as reported in similar studies (Hecker et al., 2012, 2016).

Finally, after background correction and the signal normalization, the corrected net signal was obtained by calculating the median of the duplicates and the coefficient of variation was calculated. Samples that had a CV higher than 50% were flagged and corrected. Values under

200 FI were treated as negative due to non-specific binding, and only sequences with values over 200 were considered positive for statistical analysis (Hecker et al., 2012, 2016)

### 2.11. Statistical analysis

In order to thoroughly examine the data for sequences that were significantly different between diagnostic groups and to identify epitopes that are specific for a given group (TD or ASD), we used two different analytic methods: 1) T-test- a parametric test that allowed us to compare two independent samples through mean differences and assume normal distribution of the data, and 2) Significance Analysis of Microarrays (SAM)- a permutation-based approach that measures the strength of the relationship between epitope expression and the response variable, in this case an ASD and TD diagnosis. The SAM score is directly proportional to the significance of the relationship of the data (Maximum score = 2). T-test was performed using XLSTAT 2015.1 software (Addinsoft, Paris, France), and SAM analysis was run using an R statistical computing environment. In addition, we compared the prevalence of epitope reactivity between ASD vs TD groups by Fisher exact test. Differences were considered significant if  $p < 0.05$ . Odds Ratio (OR 95% C.I) were also calculated for significant sequences using GraphPad Prism software (GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. Antigen identification

Fetal monkey brain (FMB) brain was separated by molecular weight into 110 fractions, and fraction #12 containing proteins with a molecular weight of 37–45 kDa (Fig. 1B, C) was analyzed on pairs of 2-D gels/ western blots (Fig. 2). One gel was transferred to nitrocellulose membrane and used to verify autoantibody reactivity to proteins between 37 and 45 kDa by mothers of children with ASD (Fig. 1 B and C) that were negative for the previously described autoantigens in that molecular weight range (GDA, LDHA, LDHB, and YBX1) by WB (Fig. 1 D). Multiple spots were observed, and all identified spots were collected from the second matching 2-D gel for mass spectrometric analysis (Fig. 2). Proteins near 37–45 kDa with a 100% CI were selected for verification, and detailed mass spectrometry results for the verified antigens are listed in Supplementary Table 1.

The top 4 commercially available proteins recognized by maternal autoantibodies with a 100% CI were selected for further evaluation including Neuron-Specific Enolase (NSE), Non-Specific Enolase (NSE), Fructose-Bisphosphate Aldolase C (ALDOC) and Creatinine Kinase B (CKB). Each of these proteins were tested to evaluate maternal autoantibody reactivity against individual antigens using recombinant proteins. NSE was subsequently identified by the maternal samples as corresponding to the 37–45 kDa bands, was recognized with the greatest specificity in the tested samples, and was therefore chosen as the most likely candidate for an additional MAR ASD target autoantigen.

### 3.2. Antigen verification

NSE was identified by mass spectrometry as a potential target of the maternal autoantibodies and, based on its critical role in neurodevelopment, we chose to further evaluate NSE as a



potential MAR ASD biomarker. Recombinant NSE was used to verify maternal autoantibody reactivity first by WB followed by ELISA. Reactivity was observed in 26 of the 232 mothers that had a child with ASD (6.2%) and in 21 of 186 mother that have a typically developing child (TD, 5%) suggesting that NSE alone is not a MAR ASD biomarker. Therefore, we utilized an approach similar to that used for the seven previously-described MAR autoantigens to probe the samples for differential epitope recognition between ASD and TD groups.

### 3.3. Epitope mapping

The full NSE sequence (NP\_001966.1) was translated into 434 different 15-mer peptides with 14 aa overlap and printed in duplicate onto a glass microarray, which then were probed with diluted plasma from mothers from the ASD and control groups. After the data pre-processing steps, we divided the samples into two categories based on reactivity by ELISA (Positive: samples with antibodies against NSE; Negative: samples negative to NSE in its native form but might have reactivity to cryptic epitopes) for statistical analysis. For the ELISA (+) samples, we found 16 sequences that were ASD-specific (0% TD) and 5 sequences recognized by antibodies from both groups (FI > 200). From the 16 ASD specific sequences, 4 sequences were statistically significant using both the *t*-test and SAM *t*-test (Table 2). DVAASEFYRDGKYDL ( $p = 0.047$ ; SAM score 1.49), IEDPFDQDDWAAWSK ( $p = 0.049$ ; SAM score 1.49), ERLAKYNQLMRIIEE ( $p = 0.045$ ; SAM score 1.57), and RLAKYNQLMRIIEEL ( $p = 0.017$ ; SAM score 1.82). In addition, to evaluate the association of the epitope sequences with a given group we used a Fisher exact test and found no significant differences, likely due to our small sample size. Instead, we calculated odds ratios (ORs) with 95% confidence intervals (95% CIs) for each individual peptide. We found that all ASD specific sequence had an OR above three, with SERLAKYNQLMRIIEE (OR 10.1, CI 95% 0.5094 to 200.7) and ERLAKYNQLMRIIEE (OR 12.6, CI 95% 0.6408 to 247.7) being the two epitopes with the highest OR (Fig. 3). As noted above, we found five continuous epitope sequences that were recognized by plasma from both sample groups, suggesting a large immunodominant epitope that includes the printed sequences DYPVVSIEDPFDQDD, YPVVSIEDPFDQDDW, PVSIEDPFDQDDWA, VVSIEDPFDQDDWAA, and VSIEDPFDQDDWAAW (Table 2). As is noted in Fig. 3, the sequences highlighted in red illustrate the conserved amino acids that were recognized by the antibodies in each of the five different peptide epitopes. Reactivity to the large main immunodominant epitope was also observed in ELISA (–) samples, suggesting that it is a mimotope largely recognized by general population (Table 3). Interestingly, we also found one ASD-specific epitope sequence, QDFVRDYPVVSIEDP ( $p = 0.054$ , SAM score 1.97, OR 12.6, CI 95% 0.6408 to 247.7), that was recognized by the ELISA (–) samples, suggesting that it is likely unreactive to the native structure of NSE, and more likely binding to a cryptic determinant (Table 3).

### 3.4. Bioinformatics

In order to have a better understanding of the potential origin of reactivity to the recently identified epitopes, we used the Immune Epitope Database tools (IEDB) to analyze the homology of the epitopes with all the epitopes reported in the IEDB data base. We performed a BLAST search with both 90 and 80% sequence homology settings, and found

that each of the identified sequences share homology at 90% with the other isoforms of enolase, primarily alpha enolase (Supplementary Table 2). The DYPVVSIEDPFDQDD and DYPVVSIEDPFDQDD epitopes each had 90% homology with the Protein ORF73 from Human gamma-herpesvirus 8 (Mononucleosis causing agent), and DVAASEFYRDGKYDL had 90% homology with the Outer surface protein A from *Borrelia burgdorferi* (Lyme disease causing agent). Other sequences had 80% homology with peptides from different organisms including genome polyprotein from Hepatitis C virus, virion-packaging protein UL25 from Human beta herpesvirus 6B, Alt a 6 from *Alternaria alternate*, ATP-dependent RNA helicase RhlB from *Vibrio cholerae* and Protein X from Hepatitis B virus. (Supplementary Table 2).

#### 4. Discussion

We previously described the identification of seven autoantigens recognized by autoantibodies from mothers that have a child with ASD, as well as the ASD-specific epitopes from each of the seven proteins (Braunschweig et al., 2013; Edmiston et al., 2017). The aim of the current study was to expand upon our previous work to identify additional antigens recognized by maternal autoantibodies, as well as map the unique ASD-specific NSE epitopes using microarray technology. From the proteomic analysis utilized herein, NSE emerged as a potential eighth antigen as part of the MAR ASD biomarker set. The significance of NSE as a potential MAR ASD autoantibody was based on the high antibody specificity when combined with the previously identified MAR ASD target antigens (manuscript in preparation), and the importance of NSE in neurodevelopment, brain metabolism, and brain homeostasis (Haque et al., 2018).

NSE is one of the most abundant proteins in the brain and can account for 0.4–2.2% of total soluble protein depending on the brain region. It has been implicated as having different roles including those in the glycolysis and gluconeogenesis pathways, neural cell differentiation, activation, and proliferation through the PI3K/Akt and MAPK/ERK signaling pathways. Further, NSE plays a role in the activation of the RhoA kinase pathways that can result in neurodegeneration or neuroprotection depending on the strength of the signal. In addition, NSE has been shown to be involved in CNS inflammatory processes as its expression is upregulated in MI microglia and reactive astrocytes. Therefore, NSE plays several important roles during neurodevelopment but has also been implicated in neurodegeneration (Haque et al., 2018).

Measurement of plasma NSE levels has been used as a biomarker for various applications (Isgro et al., 2015). For example, it is a useful indicator of neural maturation, and is currently the most widely used biomarker for small cell lung cancer (SCLC), and it has been shown to have a direct effect in cell growth and migration in vitro on different SCLC cell lines (Zhou et al., 2011; Liu et al., 2019). Additionally, it is also used in the diagnosis and prognosis of other type of cancers as the non-small cell lung cancer (NSCLC), neuroendocrine tumors (NETs), neuroblastoma, brain cancer and brain injury (TBI) (Isgro et al., 2015). In the present study, we addressed the value of autoantibodies to NSE as a potential biomarker of risk factor for MAR ASD based on the concept that antibody binding

to NSE during neurogenesis could impact protein functionality and brain metabolism, having a lasting impact neuronal tissue functionality and development.

In the present study, we found that autoantibody reactivity against NSE was present at similar rates for both experimental groups (ASD and TD). This indicates that the intact NSE protein is not a biomarker on its own, similar to previous studies demonstrating the necessity of autoantibody reactivity to multiple rather than single antigens to confer ASD specificity (Braunschweig et al., 2013; Singer et al., 2009; Bauman et al., 2013; Warren et al., 1990; Zimmerman et al., 2007). When we first discovered the original seven autoantigens, we found that reactivity to specific antigen combinations were highly significant as a biomarker of ASD risk, including LDH, STIP1 and CRMP1 (13% ASD vs 0% TD) and several other combinations of 3 or more autoantigens with > 98% specificity (Braunschweig et al., 2008; Braunschweig et al., 2013; Braunschweig et al., 2012). We therefore tested NSE using a larger data set and found that it increases the specificity and sensitivity of our newly improved MAR ASD assay (manuscript in preparation).

In a recent study, we performed microarray-based epitope mapping of CRMP1, CRMP2, GDA, LDHA/B, STIP1, and YBX1 and further described differential reactivity to several epitopes recognized only by autoantibodies from mothers of children with ASD (Edmiston et al., 2017). Additionally, we used the epitopes from our original set of autoantigens to create an endogenous antigen-driven mouse model for autism, in which mice were immunized with peptide epitopes for LDHA, LDHB, CRMP1 and STIP1. This methodology allowed constant exposure of the embryos to autoantibodies against the MAR ASD specific peptides throughout gestation. Hence, we created a mouse model that displayed ASD-relevant behaviors, demonstrating that exposure to this combination of autoantibodies led to alterations in neurodevelopment (Jones et al., 2018).

In the current study, we were able to identify NSE as an additional candidate MAR ASD autoantigen, and found 16 epitope sequences that are recognized by maternal autoantibodies present only in the ASD group, with 4 of those sequences demonstrating statistical significance when compared with the control group using traditional *t*-test and SAM score *t*-test analysis. Epitope Sequences (ES 408 and 409) SERLAKYNQLMRIIEE and ERLAKYNQLMRIIEE had the greatest OR values (10.1 and 12.6 respectively) indicating a strong association between having autoantibodies against these sequences and risk of having a child with ASD. However, based on the current studies, we cannot conclude that the presence of autoantibodies against those sequences would be sufficient to cause neurodevelopmental alterations. Therefore, future studies will include the use of these ASD-specific epitope peptides to create additional MAR ASD animal models. Such studies will allow us to evaluate the impact of the NSE ASD-specific peptides individually, and in combination with pathogenic epitopes from our other autoantigens, thus providing a better understanding of the role of anti-NSE in autism pathology.

As a mechanism of action, we hypothesize that the presence of autoantibodies to the ASD-specific NSE epitopes could potentially inhibit proper protein function in two different ways: 1) by directly interfering with proper protein folding (tertiary and quaternary structure) or, 2) by binding critical functional sites (catalytic or substrate sites) (Brennan et

al., 1994; Cinader and Lafferty, 1964; Lu et al., 2004; Mayes et al., 2018). While it is possible that anti-NSE antibodies in developing brain could elicit a response against cells targeted by these autoantibodies, we lack evidence of tissue destruction based on our previous rodent models. Instead, the presence of MAR ASD autoantibodies to CRMP1, LDHA/B, and STIP1 seems to affect progenitor cell maturation and alteration of adult brain dendritic spines and structure (Camacho et al., 2014; Bauman et al., 2013; Martínez-Cerdeño et al., 2014). However, the autoantibody-mediated immune pathologic mechanisms in the brain are still poorly understood and are the focus of current and future studies.

A final area of interest was exploration of the relationship between the ASD and non-ASD specific peptide sequences and the epitope repertoire reported in the Immune Epitope Database (IEDB) (Vita et al., 2019). This interest stemmed from the potential of peptide mimicry identification to provide some understanding of how autoantibodies against these self-proteins are generated. We found that sequences DYPVVSIEDPFDQDD, YPVVSIEDPFDQDDW, PVVSIEDPFDQDDWA, VVSIEDPFDQDDWAA, VSIEDPFDQDDWAAW are recognized by antibodies in both experimental groups indicating an immunodominant epitope recognized by the general population. As anticipated, these sequences share a high degree of homology with alpha and gamma enolase (NNE and NSE) at 90% stringency, and interestingly share 80% homology with other proteins including Protein ORF73 from human gamma-herpesvirus 8 (Mononucleosis causing agent), Protein X from Hepatitis B virus and Serpin H1 from humans indicating possible molecular mimicry to direct exposure to these agents. However, due to the significant limitations of only comparing linear sequences and not taking into account potential conformational epitopes and MHC presentation dynamics, we have no means to conclude that the anti-NSE antibodies are in fact produced in response to infection with any of the mentioned agents, or because of tissue damage and epitope spreading of Serpin H1.

It is important to mention the limitations of the current study. First, a relatively small sample size was used for the microarray studies. Secondly, microarray artifacts such as array printing and handling can occur during testing and imaging. However, we used an enhanced data pre-processing method (Zhu et al., 2006) that allowed us to generate uniform data and enabled us to identify significant ASD-specific NSE epitopes despite our small sample size. The third limitation, as mentioned above is that the bioinformatics tools utilized herein considered only linear epitopes and did not take in account conformational epitopes or MHC presentation, which are key factors in the generation of an immune response. However, from the current study we can conclude that NSE is a target of autoantibodies, and there are specific epitopes recognized by antibodies from the ASD group exclusively, making these epitopes an interesting potential biomarker for MAR ASD. Ongoing studies include investigation of the pathogenic mechanisms of MAR-related autoantibodies using animal models including mice, rats, and non-human primates, as well as additional in silico modeling studies of the epitope-protein interaction for identification of the key amino acids that ascribe functional significance.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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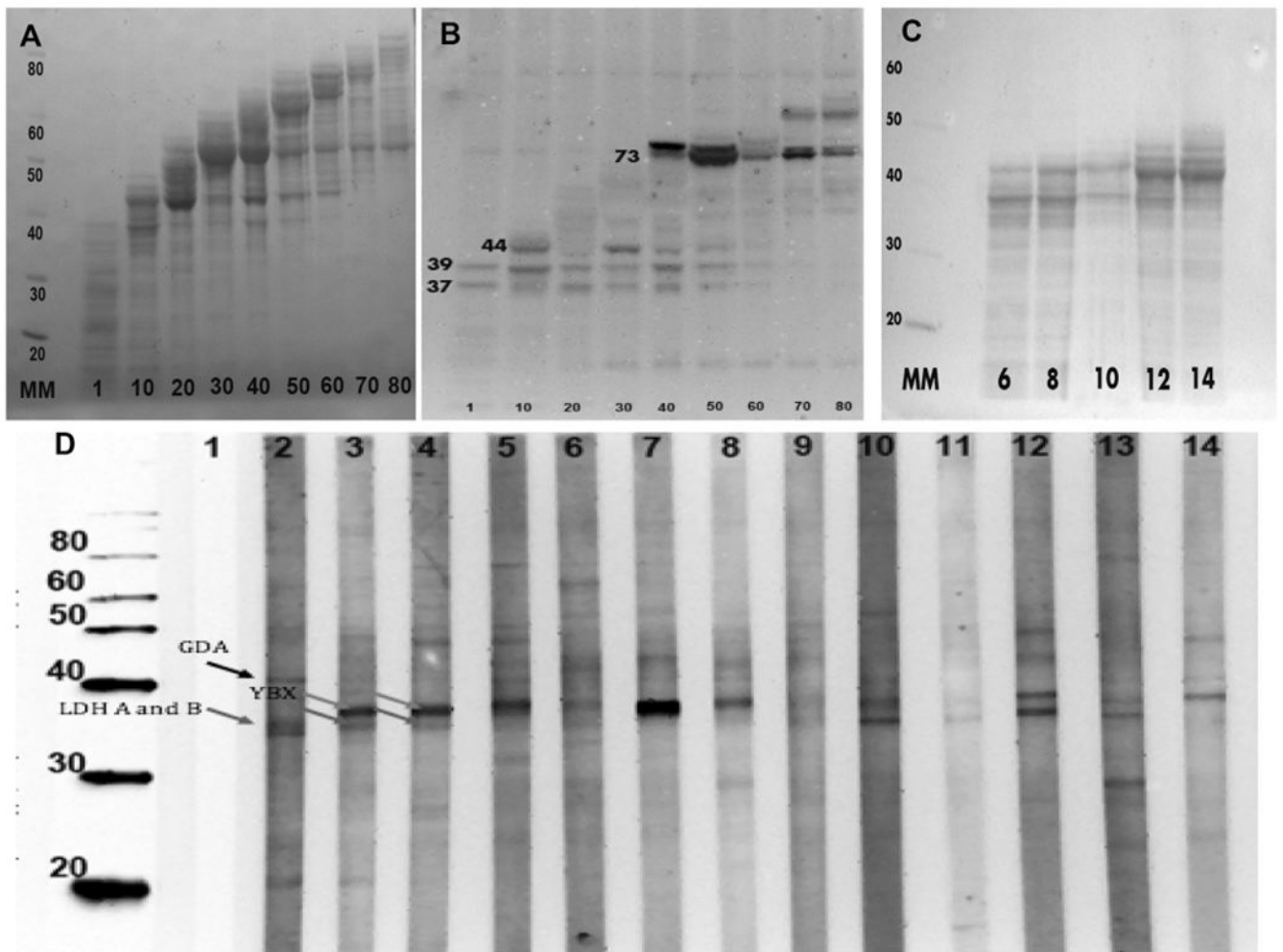
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**Fig. 1.** Western blot (WB) of fetal monkey brain (FMB) probed with maternal plasma. (A) Ponceau stained nitrocellulose membrane containing samples from the first fraction, and every tenth fraction thereafter, collected from the Prep Cell separation of FMB. (B) WB of the duplicated membrane shown in (A) probed with a pool of maternal plasma reactive to 37 kDa (LDH), 39 kDa (YBX1), 44 kDa (GDA), and 73 kDa (STIP1, CRMP1/2) antigens. (C) Prep Cell fractions, which contained proteins between 39 and 42 kDa. Fraction # 12 was used for 2D gel electrophoresis. (D) WB of FMB fraction #12 probed with maternal plasma not reactive to LDHA-B, GDA and YBX1. Lane 1: secondary-only antibody control, Lanes 2–4: maternal plasma reactive to LDHA-B (Green Arrows), YBX1 (Blue Arrows), and GDA (Black Arrow). Lanes 5–8 maternal plasma pool #1 and with band reactivity to a protein near 39 kDa. Lanes 10–14: maternal plasma pool #2 with band reactivity to two proteins near 37 and 39 kDa. Lane 9. Plasma sample negative-control to FMB antigens. Abbreviations: FMB, fetal monkey brain; LDHA-B, lactate dehydrogenase A and B; YBX1, Y-box binding protein 1; GDA, guanine deaminase; CRMP1 and CRMP2, collapsin response mediator 1 and 2, and STIP1, stress induced phosphoprotein 1. (For interpretation

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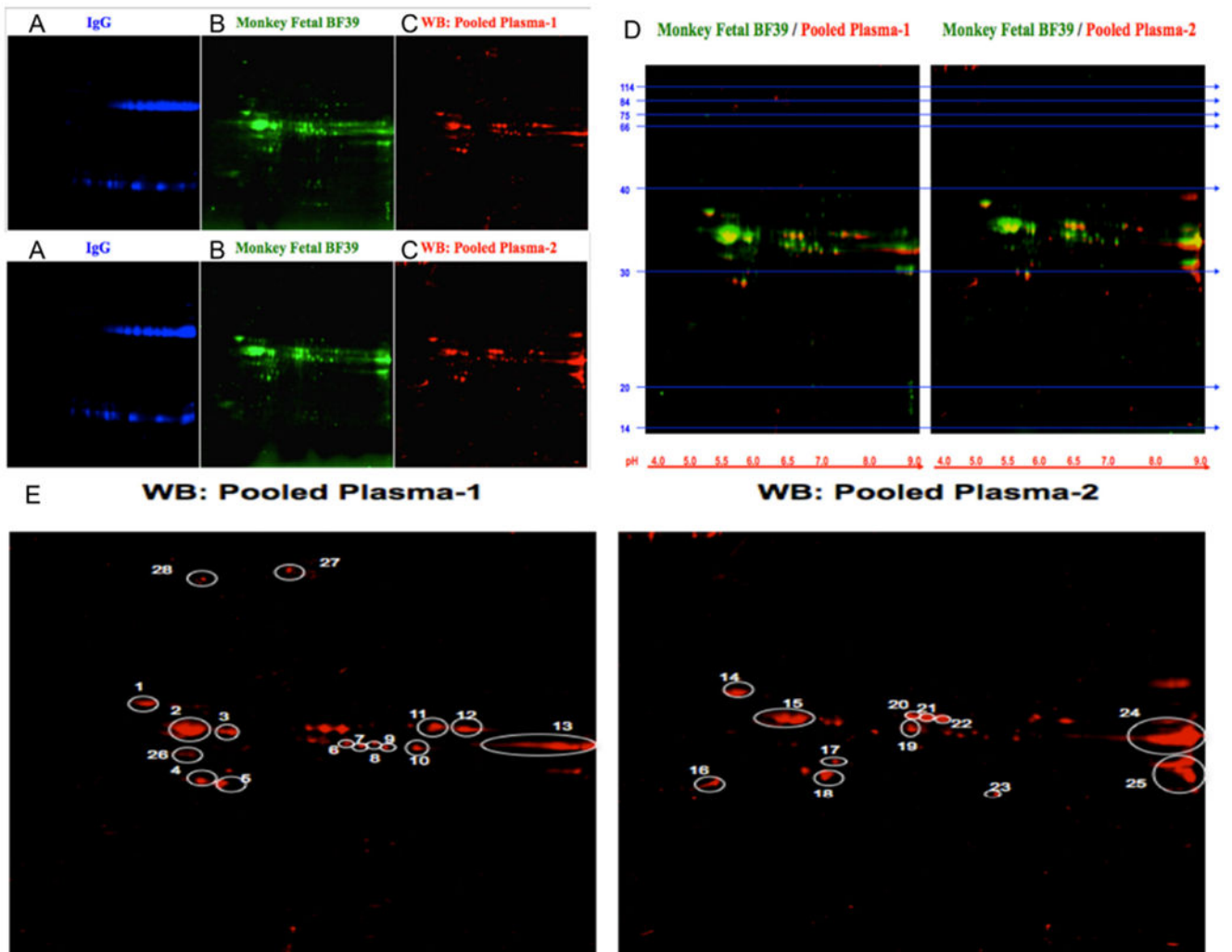
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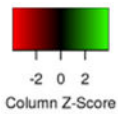
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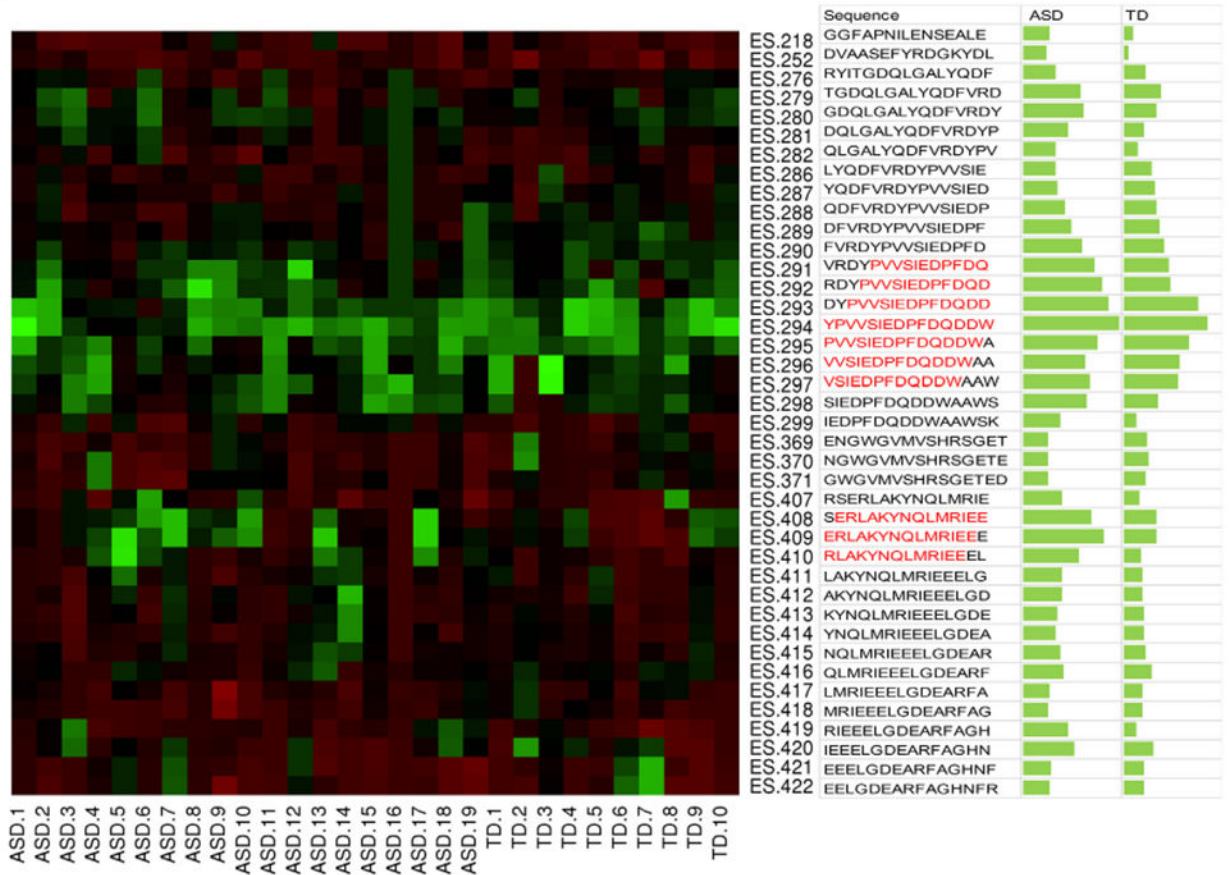




**Fig. 2.** Two-dimensional (2-D) gel electrophoresis and antigen selection for mass spectrometry. (A) depicts anti-IgG stained gel for protein alignment with FMB fraction #12 (B) and the membraned blotted with plasma pool 1 and plasma pool 2 (C). (D) depicts the merged images of B and C. (E) WB of the proteins that were bound by maternal IgG antibodies (Pooled plasma 1 and 2), each of which was labeled with a spot number. In total, 27 protein spots were picked and subsequently analyzed by mass spectrometry.



ELISA POSITIVE



**Fig. 3.** Heat map of sequences with average reactivity (FI) over 50 from ELISA positive samples. Samples were considered positives if FI > 200. Letters in red illustrate the amino-acid residues that are part of the main epitope in ES 293-297, and ES 408-410 illustrate the amino acid sequences recognized by the ASD group only. On the right, a histogram represents the FI reactivity. Abbreviations: ES, Epitope Sequence; ASD, Autism Spectrum Disorders; TD, Typically Developing; FI, Fluorescence Intensity.

**Table 1**

Demographics of study population. Illustrates the mean maternal age at birth of child and mean age of child at time of sample collection.

Diagnosis	Number of Subjects	Maternal Age at birth of child (yrs)			Child Age at time of draw (mo)		
		SD	Max	Min	SD	Max	Min
ASD	28	30	40	19	49	9	31
ELISA +	20						
ELISA –	8						
TD	22	31	36	20	46	8	25
ELISA +	11						
ELISA –	11						

Abbreviations: ASD, Autism Spectrum Disorders; TD, Typically Developing; SD, Standard Deviation, Max, Maximum age, Min, Minimum age.

<sup>a</sup>Subjects from Childhood Autism Risk from Genetics and the Environment (CHARGE) study (Hertz-Picciotto et al., 2006).

**Table 2**  
Summary of significant NSE epitopes recognized by maternal autoantibodies (ELISA positive).

ES#	Sequence	ASD + . N = 19	TD + . N = 10	p-value	T-test	SAM score	t-test	Fisher's exact test p-value	Odds Ratio	Confidence Interval (95%)	Specific binding
218	GGFAPNILENSEALE	3	0	0.163		0.94		0.532	4.455	0.2082 to 95.32	ASD
252	DVAASEFYRDGKYDL	3	0	<b>0.047</b>		<b>1.49</b>		0.532	4.455	0.2082 to 95.32	ASD
279	TGDLGALYQDFVRD	5	0	0.295		0.77		0.134	7.966	0.3955 to 160.4	ASD
280	GDQLGALYQDFVRDY	5	0	0.101		1.23		0.134	7.966	0.3955 to 160.4	ASD
281	DQLGALYQDFVRDYP	3	0	0.060		1.37		0.532	4.455	0.2082 to 95.32	ASD
289	DFVRDYPVVSIEDPF	3	0	0.459		0.47		0.532	4.455	0.2082 to 95.32	ASD
298	SIEDPFQDDWAAWS	4	0	0.093		1.23		0.268	6.097	0.2958 to 125.6	ASD
299	IEDPFQDDWAAWSK	2	0	<b>0.049</b>		<b>1.49</b>		0.532	3.000	0.1309 to 68.76	ASD
407	RSERLAKYNQLMRIE	3	0	0.079		1.25		0.532	4.455	0.2082 to 95.32	ASD
408	SERLAKYNQLMRIEE	6	0	0.107		1.25		0.068	10.110	0.5094 to 200.7	ASD
409	ERLAKYNQLMRIEEE	7	0	<b>0.045</b>		<b>1.57</b>		0.063	12.600	0.6408 to 247.7	ASD
410	RLAKYNQLMRIEEEL	5	0	<b>0.017</b>		<b>1.82</b>		0.134	7.966	0.3955 to 160.4	ASD
411	LAKYNQLMRIEELG	3	0	0.109		1.12		0.532	4.455	0.2082 to 95.32	ASD
412	AKYNQLMRIEEELGD	3	0	0.125		1.07		0.532	4.455	0.2082 to 95.32	ASD
413	KYNQLMRIEEELGDE	3	0	0.231		0.81		0.532	4.455	0.2082 to 95.32	ASD
416	QLMRIEEELGDEARF	3	0	0.234		0.83		0.532	4.455	0.2082 to 95.32	ASD
293	DYPVVSIEDPFQDD	6	4	0.711		0.3		0.698	0.692	0.1408 to 3.405	ASD and TD
294	YPVVSIEDPFQDDW	8	4	0.650		0.39		1.000	1.091	0.2294 to 5.187	ASD and TD
295	PVVSIEDPFQDDWA	5	2	0.643		0.3		1.000	1.429	0.2232 to 9.142	ASD and TD
296	VVSIEDPFQDDWAA	4	1	0.793		0.17		0.632	2.400	0.2306 to 24.98	ASD and TD
297	VSIEDPFQDDWAAW	4	1	0.602		0.44		0.632	2.400	0.2306 to 24.98	ASD and TD

Abbreviations: ES, Epitope Sequence; ASD, Autism Spectrum Disorders; TD, Typically Developing. Bold font indicates statistical significance.

**Table 3**

Summary of significant NSE epitopes recognized by maternal autoantibodies (ELISA negative).

ES #	Sequence	ASD + . N = 8	TD + . N = 11	p-value T-test	SAM score t-test	Fisher's exact test p-value	Odds Ratio	Confidence Interval (95%)	Specific binding
288	QDFVRDYPVVSIEDP	3	0	<b>0.054</b>	<b>1.97</b>	0.069	13.360	0.5793 to 308.3	ASD
290	FVRDYPVVSIEDPFD	3	1	0.174	1.34	0.275	5.400	0.4371 to 66.71	ASD and TD
291	VRDYPVVSIEDPFDQ	4	4	0.695	0.3	1.000	1.500	0.2296 to 9.801	ASD and TD
292	RDYPVVSIEDPFDQD	5	2	0.455	0.8	0.145	6.667	0.8083 to 54.99	ASD and TD
293	DYPVVSIEDPFDQDD	6	7	0.307	1.02	0.638	2.000	0.2599 to 15.39	ASD and TD
294	YPVVSIEDPFDQDDW	5	7	0.388	0.8	1.000	1.111	0.1644 to 7.510	ASD and TD
295	PVVSIEDPFDQDDWA	4	5	0.697	0.24	1.000	1.500	0.2296 to 9.801	ASD and TD
296	VVSIEDPFDQDDWAA	4	2	0.111	1.7	0.321	4.000	0.5000 to 32.00	ASD and TD
297	VSIEDPFDQDDWAAW	2	2	0.266	1.2	1.000	1.333	0.1436 to 12.38	ASD and TD

Abbreviations: ES, Epitope Sequence; ASD, Autism Spectrum Disorders; TD, Typically Developing. Bold font indicates statistical significance.