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Systematic Characterization of the Immune Response to Gluten and Casein in Autism Spectrum Disorders

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14. ABSTRACT Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental diseases characterized by deficits in communication skills and social interaction, as well as the presence of repetitive and stereotyped patterns of behavior. The reported prevalence of ASD has increased sharply over the last few decades to greater than 1%, while the pathogenic mechanisms of the disease remain largely unknown and effective treatment options are limited. In addition, a clear barrier to the better understanding of ASD has been the heterogeneity within this spectrum and the lack of biomarkers to characterize disease phenotypes and to understand treatment outcome. Dietary gluten and casein proteins have been suspected of being involved in the etiopathogenesis of ASD in some patients, either directly as circulating partially digested peptides with opioid-like properties, or through the body's immune response to them. Diets that exclude gluten and casein have been reported to be effective in some cases. However, significant methodological shortcomings in some of these reports and contradicting data from other investigators have contributed to a lack of consensus and a high level of ambiguity on the actual existence of these molecules in the body and their relevance to ASD. We have utilized serum samples from well-characterized patients diagnosed with autism according to strict criteria (both ADOS and ADI-R), their unaffected siblings, and age-matched unrelated healthy controls to assess immune reactivity to highly purified gluten and casein molecules or peptides and to evaluate the potential link between autism and celiac disease. Our data show that a subset of children with autism displays increased immune reactivity to gluten and casein, which is associated with presence of gastrointestinal symptoms, but not with celiac disease. The mechanism of the immune response to gluten in autism is fundamentally different from that in celiac disease, being independent of TG2 activity and antigen presentation by HLA-DQ2/DQ8 molecules. The increased anti-gliadin antibody response and its association with GI symptoms point to a potential mechanism involving immunologic and/or intestinal permeability abnormalities in affected children. In addition, the data point to potential opportunities for identifying novel antibody biomarkers that may identify a subset of patients who would benefit from specific therapeutic interventions.					
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INTRODUCTION:

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental diseases characterized by deficits in communication skills and social interaction, as well as the presence of repetitive and stereotyped patterns of behavior. The reported prevalence of ASD has increased sharply over the last few decades to greater than 1%, while the pathogenic mechanisms of the disease remain largely unknown and effective treatment options are limited. In addition, a clear barrier to the better understanding of ASD has been the heterogeneity within this spectrum and the lack of biomarkers to characterize disease phenotypes and to understand treatment outcome. Dietary gluten and casein proteins have been suspected of being involved in the etiopathogenesis of ASD in some patients, either directly as circulating partially digested peptides with opioid-like properties, or through the body's immune response to them. Diets that exclude gluten and casein have been reported to be effective in some cases. However, significant methodological shortcomings in some of these reports and contradicting data from other investigators have contributed to a lack of consensus and a high level of ambiguity on the actual existence of these molecules in the body and their relevance to ASD. We have utilized serum samples from well-characterized patients diagnosed with autism according to strict criteria (both ADOS and ADI-R), their unaffected siblings, and age-matched unrelated healthy controls to assess immune reactivity to highly purified gluten and casein molecules or peptides and to evaluate the potential link between autism and celiac disease.

BODY:

This report summarizes the work conducted over the entire research period, cites data in annual reports and appended publications. The report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work.

Aim 1. To characterize the prevalence and specificity of the anti-gluten and anti-casein immune response in ASD.

Task A. Serologic markers of gluten sensitivity and celiac disease in children with autism

The work in this section was reported in the first (partial data) and second annual reports. The data in this section were published in 2013 (Lau et al., PLOS ONE 2013; 8(6):e66155) [1].

Serum samples. Serum samples from 140 children were analyzed, including 37 with autism, 27 unaffected siblings of similar ages within the same families, and 76 unrelated healthy controls. The sera from individuals with autism and their siblings were acquired from the Autism Genetic Resource Exchange (AGRE). All affected children met the diagnostic criteria for autism based on both the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview, Revised (ADI-R) (resulting in the above stated total number of available serum samples from AGRE at the time of selection (4/29/2010)). In addition, the Vineland Adaptive Behavior Scales was performed during the diagnostic evaluation. Medical histories and physical examinations were conducted by pediatric neurologists, psychologists, and developmental specialists affiliated with AGRE. Information on GI symptoms was based on parent questionnaires, interviews, and medical histories. The data collected by AGRE from these evaluations were retrieved from the online AGRE phenotype database. The control sera were from healthy children in the United States (n=14) and Sweden (n=62). The healthy controls from U.S. had been recruited in a general pediatric clinic at the Weill Cornell Medical College in New York City. The healthy controls from Sweden had been recruited at child health care centres and schools in the Falun region in central Sweden [2]. Screening questionnaires were used to evaluate the general health of the U.S. and Swedish controls. Individuals who had reported having a chronic disease, allergy, or recent infection were not included. Serum from a celiac disease patient, diagnosed according to previously described criteria [3], was used as a positive control for the anti-gluten antibody assays. All serum samples from patients and control individuals had been anonymized and were maintained at -80 °C prior to testing. This study was approved by the Institutional Review Board of Columbia University Medical Center.

The demographic and clinical characteristics of the patients with autism, their unaffected siblings, and unrelated healthy controls are shown in Table 1. The patient cohort included four individuals on gluten-free diet. Because the effect of the exclusion diet on IgG antibody level in autism is unknown, these were not excluded from the analysis.

Gluten. The antigen used for

Table 1. Demographic characteristics of study cohorts.

Subject group	Number of subjects	Mean age— years ± SD	Male sex— no. (%)	White race— no. (%)
Autism	37	7.8 ± 2.9	29 (78)	33 (89)
With GI symptoms	19	7.1 ± 2.3	13 (68)	15 (79)
Without GI symptoms	8	7.1 ± 2.3	6 (75)	8 (100)
Unaffected sibling	27	8.1 ± 2.9	18 (67)	25 (93)
Unrelated healthy	76	8.8 ± 3.7	59 (77)	70 (92)

the anti-gluten antibody assays was the Prolamine Working Group (PWG) reference gliadin, which was extracted from a combination of 28 different wheat varieties, as previously described [4]. The protein profile of the PWG gliadin extract was assessed by SDS-polyacrylamide gel electrophoresis, using 10% NuPAGE Bis-Tris precast gels and 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (Life Technologies, Carlsbad, Calif.). The gel electrophoresis profile of the PWG gliadin used for the anti-gluten antibody assays indicated the presence of all main types of gliadin proteins, α/β , γ , and ω . The mixture also contained high and low molecular weight glutenin subunits (Fig. 1).

Antibody levels. Serum IgG and IgA antibodies to gluten were measured by enzyme-linked immunosorbent assay (ELISA), similarly to previously described procedure [5]. A 2 mg/mL stock solution of the PWG gliadin was prepared in 60% ethanol. 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 μ L/well of a 0.01 mg/mL solution of PWG gliadin in 0.1 M carbonate buffer (pH 9.6) or were left uncoated to serve as control wells. After incubation at 37 °C for 1 h, all wells were washed and blocked by incubation with 1% bovine serum albumin (BSA) in phosphate buffered saline containing 0.05% Tween-20 (PBST) for 1.5 h at room temperature. Serum samples were diluted at 1:800 for IgG measurement and at 1:200 for IgA measurement, added at 50 μ L/well in duplicates, and incubated for 1 h. Each plate contained a positive control sample from a patient with biopsy-proven celiac disease and elevated IgG and IgA antibodies to gluten. After washing the wells, they were incubated with HRP-conjugated sheep anti-human IgG (GE Healthcare, Piscataway, N.J.) or IgA (MP Biomedicals, Santa Ana, Calif.) secondary antibodies for 50 min. The plates were washed and 50 μ L of developing solution, comprising 27 mM citric acid, 50 mM Na_2HPO_4 , 5.5 mM *o*-phenylenediamine, and 0.01% H_2O_2 (pH 5), was added to each well. After incubating the plates at room temperature for 20 min, absorbance was measured at 450 nm. All samples were tested in duplicates. Absorbance values were corrected for non-specific binding by subtraction of the mean absorbance of the associated BSA-coated wells. Data for all serum samples were normalized to the mean value of the positive control duplicate on each plate. The mean level of antibody for the unrelated healthy control cohort was set as 1.0 AU and all other results were normalized to this value.

IgA antibody to recombinant human TG2 was measured in all sera using an ELISA kit, according to the manufacturer's protocol (Euroimmun AG, Lubeck, Germany). Sera were tested for IgG and IgA antibody reactivity to a previously described glutamine-glutamate substituted trimer of a fusion peptide containing the sequences PLQPEQPFP and PEQLPQFEE [6]. Antibodies were measured using an ELISA kit, according to the manufacturer's protocol (Euroimmun AG).

Differences between groups were analyzed by the two-tailed Student's *t* test, Welch's *t* test, Mann-Whitney U test, or one-way analysis of variance (ANOVA) with post-hoc Dunn test (continuous data), and the Fisher's exact test (nominal data). Adjustment for covariate effect (age, gender, and race) was carried out by analysis of covariance (ANCOVA), using the general linear model. Logistic regression was used to calculate the odds ratios associated with increased antibodies in individuals with autism. For these analyses, increased levels of anti-gluten antibody were defined as values at 95th percentile or higher of the unrelated healthy control group. For IgA anti-TG2 antibody and IgG/IgA anti-deamidated gliadin antibodies, cutoffs for positivity were assigned by the manufacturer. Differences with *p* values of <0.05 were considered to be statistically significant. Statistical analyses were performed with Prism

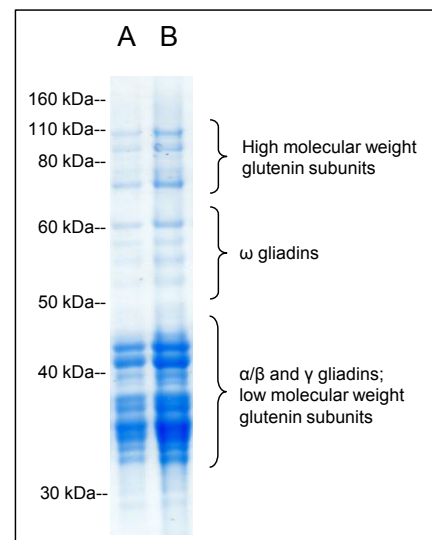


Figure 1. Gel electrophoresis profile of the PWG gliadin preparation used for the anti-gluten antibody assays. A) 5 μ g of protein loaded; B) 10 μ g of protein loaded.

5 (GraphPad, San Diego, Calif.) and Minitab 16 (Minitab, State College, Pa.).

Mean levels of IgG and IgA class antibodies to gluten in patient and control groups are presented in Fig. 2. Children with autism exhibited significantly elevated levels of IgG antibody to gluten when compared with non-relative healthy controls or when compared with the combination of unaffected siblings and non-relative healthy controls (non-parametric one-way ANOVA with post-hoc Dunn test, $p < 0.01$). The difference remained significant even after adjusting for the covariates of age, gender, and race (ANCOVA with general linear model, $p < 0.01$). The anti-gluten IgG differences between the autistic cohort and siblings, and between the siblings and unrelated healthy controls, did not reach statistical significance. Based on the stated cutoff for positivity (95th percentile of the healthy control group), 8/37 (21.6%) of autistic children, 2/27 (7.4%) of unaffected siblings, and 4/76 (5.3%) of unrelated healthy children were positive for IgG anti-gluten antibody, representing a significantly higher frequency in autistic children compared to unrelated healthy controls (Fisher's exact test, $p < 0.05$). Children with autism had increased odds of having elevated IgG antibody to gluten in comparison to healthy controls (odds ratio: 4.97; 95% confidence interval: 1.39-17.8). The differences in levels of IgA antibody to gluten among the three groups were not significant.

Elevated IgG antibody reactivity to gluten was confirmed by immunoblotting in all antibody-positive patients.

None of the individuals in any group were positive for IgA antibody to TG2. Two of the 37 of autistic children, 3 of the 27 of unaffected siblings, and none of the 76 unrelated healthy controls had values above the manufacturer's assigned cutoff for IgG antibody to deamidated gliadin. Similarly, none of the 37 autistic children, 1 of the 27 of unaffected siblings, and 1 of the 76 of unrelated healthy controls were positive for IgA antibody to deamidated gliadin.

All four individuals who were on gluten-free diet were negative for anti-gluten, anti-deamidated gliadin, and anti-TG2 antibodies.

GI symptoms. Medical histories were available for 27 of the 37 children with autism. 19/27 (70.3%) reported GI symptoms, including 10 with chronic loose stools or diarrhea, 2 with gastroesophageal reflux, 3 with frequent stools, 3 with constipation, and 1 with non-specified GI symptoms. Affected patients with GI symptoms were found to have significantly higher levels of IgG antibody to gluten when compared to patients without GI symptoms (Welch's t test, $p < 0.01$) (Fig. 3A). This difference remained significant after adjusting for the covariates of age, gender, and race (ANCOVA with general linear model, $p < 0.01$).

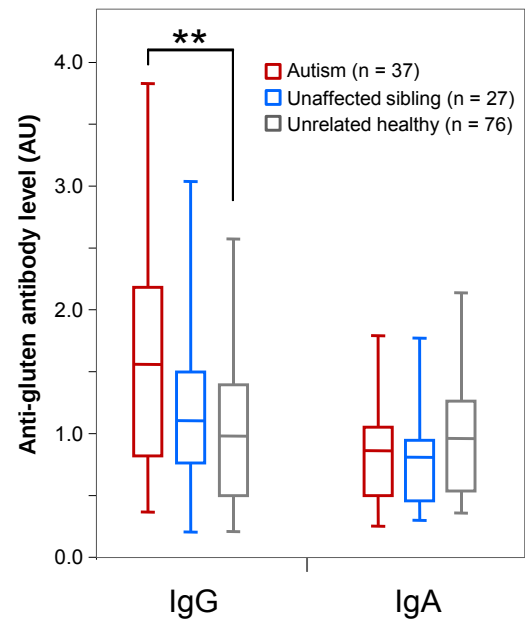


Figure 2. Comparison of mean levels of IgG and IgA antibody to gluten in children with autism, their unaffected siblings, and unrelated healthy controls. Boxed segments represent the middle 50% of the data. Whiskers indicate the range of data. Large horizontal bars indicate mean value of the data. ** = $p < 0.01$. Based on the stated cutoff for positivity (95th percentile of the healthy control group), 8/37 (21.6%) of autistic children, 2/27 (7.4%) of unaffected siblings, and 4/76 (5.3%) of unrelated healthy children were positive for IgG anti-gluten antibody

There was no significant difference in the levels of IgA antibody to gluten (Fig. 3A), IgG and IgA antibodies to deamidated gliadin (Fig. 3B), or IgA antibody to TG2 (Fig. 3C) between patients with GI complaints and those without. One autism patient with GI symptoms was positive for IgG antibody to deamidated gliadin, while the remaining patients in both groups were negative for all other markers.

Task B. Immune response to casein in autism

Serum samples. Serum samples from 184 children were analyzed, including 70 with autism, 36 unaffected siblings of similar ages within the same families, and 78 unrelated healthy controls. Serum samples were acquired from the Autism Genetic Resource Exchange (AGRE) (37 autism and 28 unaffected siblings) and the Weill Cornell Autism Research Program (WCARP) (33 autism, 8 unaffected siblings, and 16 unrelated healthy controls). AGRE patients met diagnostic criteria for autism based on both the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview, Revised, while WCARP patients met criteria for autism according to ADOS.

Casein. Antibody reactivity to each of the three types of casein, α -casein, β -casein, and κ -casein (Sigma-Aldrich), was assessed separately in all available serum samples. The protein profile of the each casein extract was assessed by SDS-polyacrylamide gel electrophoresis, as was described above and can be seen in Fig. 4.

Antibody levels. Serum IgG and IgA antibodies to α -casein, β -casein, and κ -casein were measured by enzyme-linked immunosorbent assay (ELISA), similarly to previously described procedure [5]. 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 μ L/well of a 0.01 mg/mL solution of α -casein, β -casein, or κ -casein in 0.1 M carbonate buffer (pH 9.6) or were left uncoated to serve as control wells. After incubation at 37 °C for 1 h, all wells were washed and blocked by incubation with 1% bovine serum albumin (BSA) in phosphate buffered saline containing 0.05% Tween-20 (PBST) for 1.5 h at room temperature. Serum samples were diluted at 1:1000 for IgG measurement and at 1:250 for IgA measurement, added at 50 μ L/well in duplicates, and incubated for 1 h. Each plate contained a positive control sample from an individual with elevated antibody reactivity to casein. After washing the wells, they were incubated with HRP-conjugated sheep anti-human IgG (GE Healthcare, Piscataway, N.J.) or IgA (MP Biomedicals, Santa Ana, Calif.) secondary antibodies for 50 min. The plates were

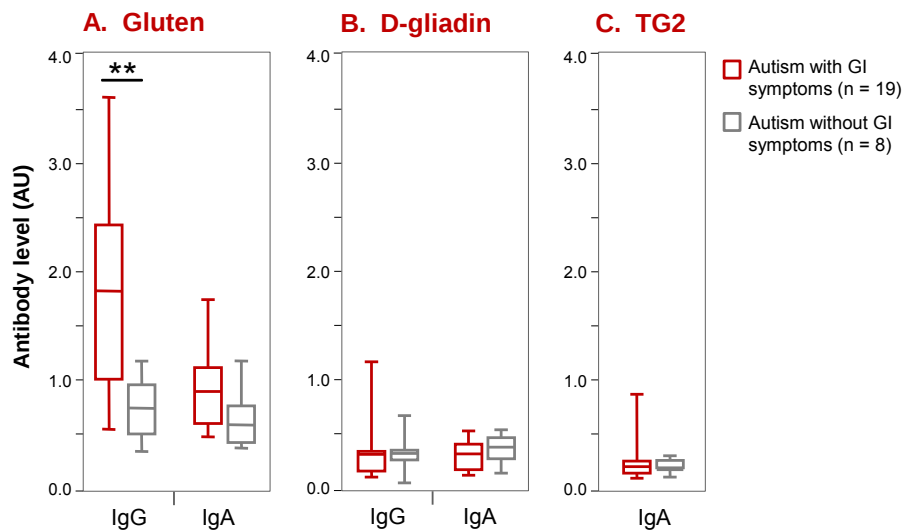


Figure 3. Comparison of mean levels of antibody to A) gluten, B) deamidated gliadin fusion peptide, and C) human TG2 in autistic children, with and without GI symptoms. Boxed segments represent the middle 50% of the data. Whiskers indicate the range of data. Large horizontal bars indicate mean value of the data. ** = $p < 0.01$.

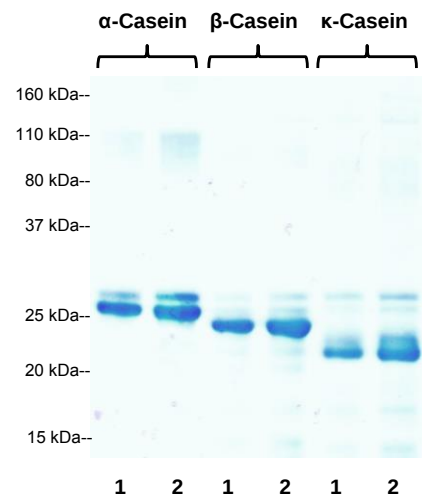


Figure 4. Gel electrophoresis profile of the α , β , and κ -casein preparations used for the anti-casein antibody assays. 1) 5 μ g of protein loaded; 2) 20 μ g of protein loaded.

washed and 50 μ L of developing solution, comprising 27 mM citric acid, 50 mM Na_2HPO_4 , 5.5 mM o-phenylenediamine, and 0.01% H_2O_2 (pH 5), was added to each well. After incubating the plates at room temperature for 20 min, absorbance was measured at 450 nm. All samples were tested in duplicates. Absorbance values were corrected for non-specific binding by subtraction of the mean absorbance of the associated BSA-coated wells. Data for all serum samples were normalized to the mean value of the positive control duplicate on each plate. The mean level of antibody for the unrelated healthy control cohort was set as 1.0 AU and all other results were normalized to this value. Statistical analysis was carried out as in section A.

Children with autism exhibited significantly elevated levels of IgG antibody to α -casein ($p < 0.001$), β -casein ($p < 0.001$), and κ -casein ($p < 0.01$) when compared with non-relative healthy controls. The difference remained significant even after adjusting for the covariates of age, gender, and race (ANCOVA with general linear model). The anti-gluten IgG differences between the autistic cohort and siblings did not reach statistical significance.

Based on the stated cutoff for positivity (95th percentile of the healthy control group), 7/70 (10.0%) of autistic children, 2/38 (5.3%) of unaffected siblings, and 4/78 (5.1%) of unrelated healthy children were positive for IgG anti- α -casein antibody, not representing a significantly higher frequency in autistic children compared to unrelated healthy controls. With regard to anti- β -casein IgG antibodies, 12/70 (17.1%) of autistic children, 3/36 (8.3%) of unaffected siblings, and 4/78 (5.1%) of unrelated healthy children were positive, representing a significantly higher frequency in autistic children compared to unrelated healthy controls (Fisher's exact test, $p < 0.05$). With regard to anti- κ -casein IgG antibodies, 15/70 (21.4%) of autistic children, 5/36 (13.9%) of unaffected siblings, and 4/78 (5.1%) of unrelated healthy children were positive, representing a significantly higher frequency in autistic children compared to unrelated healthy controls (Fisher's exact test, $p < 0.01$). The differences in frequencies of IgA antibody to gluten among the three groups were not significant.

Elevated antibody reactivity to casein was confirmed by immunoblotting in all antibody-positive patients (Fig. 6)

Children with autism had increased odds of having elevated IgG antibody to β - and κ -casein in comparison to healthy controls (odds ratio: 3.83, 95% confidence interval: 1.17-12.5 for β -casein; odds ratio:

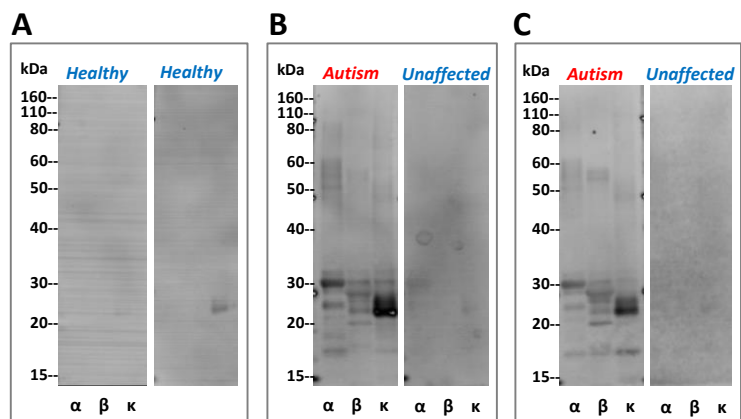
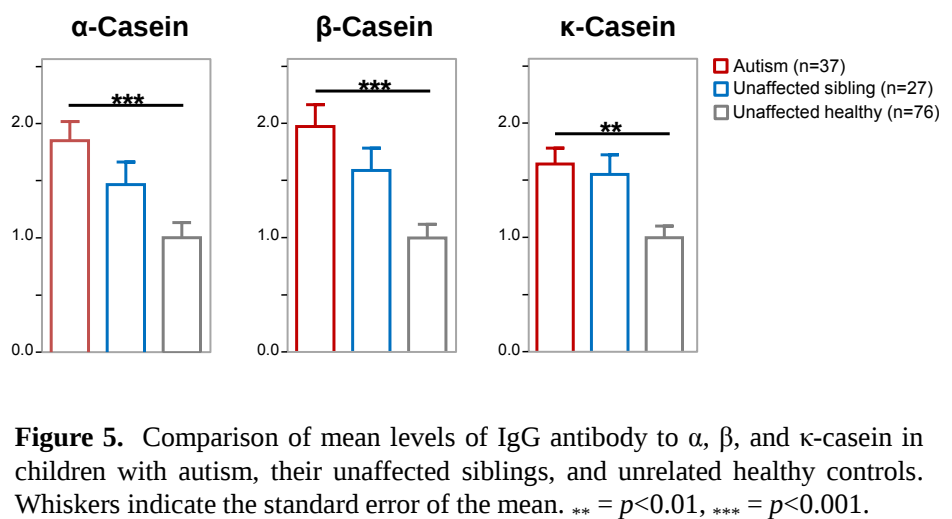


Figure 6. Western blot patterns of IgG antibody reactivity to α -, β -, and κ -casein in representative patients and controls. A) Two representative unrelated healthy controls, B) a representative patient with autism and elevated antibodies and his unaffected sibling, C) a representative patient with autism and elevated antibodies and his unaffected sibling.

5.04, 95% confidence interval: 1.59-16.1 for κ -casein).

There was a significant correlation between the levels of antibody to casein and antibodies to gluten ($p < 0.05$).

Task C. Specificity of antibody response to gluten and casein.

To confirm that the observed increase in anti-gliadin and anti-casein antibodies were not non-specific IgG responses, sera were tested for antibodies to another group of foreign antigens (*Borrelia burgdorferi* proteins), as well as to a nervous system autoantigen (GM1 ganglioside). There was no difference in IgG anti-*B. burgdorferi* or anti-GM1 antibodies between patients and controls [7,8], nor was there any correlation between these and anti-gluten/anti-casein antibodies, indicating that the detected antibody response to gluten and casein are not part of a general increased IgG antibody response in autism.

Aim 2. To assess serum levels of casein and gluten exorphin peptides and the immune response to them in ASD individuals.

Task A. Levels of casein and gluten exorphins in serum.

Samples were to be analyzed for specific gluten and casein exorphin peptides by competitive immunoassays. As indicated in the statement of work, these assays involve 20-fold concentration of serum, requiring at least 500 μ L of neat serum. However, despite tremendous effort on our part, we were unable to acquire enough serum volume for these assays. The price offered by AGRE at the time of acquisition (50 μ L for \$50) made the acquisition of the required number of samples impractical. Other sources either did not have or were not willing to give us the amount serum we required.

Task B. Levels of antibody reactivity to casein and gluten exorphins in serum.

Apart from the lack of enough serum volume for the above experiments in Task A, a shortcoming of the strategy of detecting peptides in circulation is that levels are going to depend largely on what each subject has consumed in the last 1-2 days (due to degradation and renal clearance). Therefore, considering that we could not control for subject diet, we planned to also examine the potential antibody response to specific sequences containing the described peptides.

Serum samples. Serum samples from 30 children were analyzed, including 7 with autism and elevated antibody to gliadin, 20 with autism and no antibody reactivity to gliadin, and 14 age-matched healthy controls. Serum samples were acquired from AGRE and WCARP, as described in Aim1 tasks.

Exorphins. Antibody reactivity to gluten exorphins A5 (GYYPYPT), B5 (YGGWL), and gliadorphin 7 (YPQPQPF), as well as the casein peptides β -casomorphin 7 (BCM7) (YPFPGPI), and morphiceptin (YPFP) was assessed separately. The peptides were synthesized by utilizing Fmoc chemistry (Sigma-Aldrich, St. Louis, Missouri).

Antibody levels.

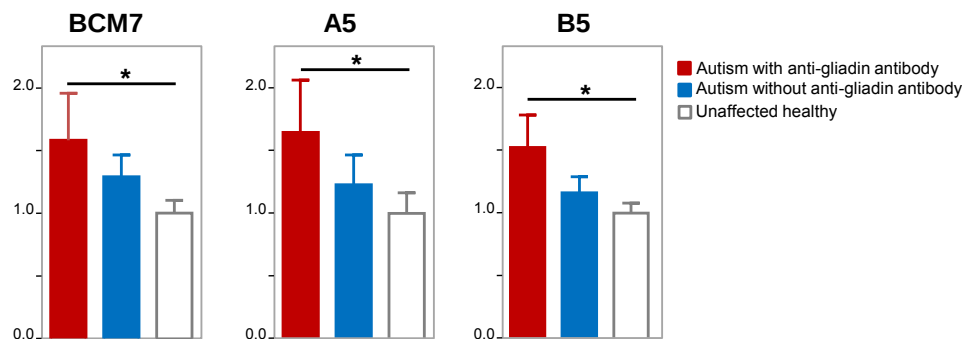


Figure 7. Comparison of mean levels of IgG antibody to BCM7, A5, and B5 exorphin peptides in children with autism and elevated antibody to gliadin, autistic children without antibody to gliadin, and unrelated healthy controls. Whiskers indicate the standard error of the mean. * = $p < 0.05$.

Serum IgG and IgA antibodies to the above peptides were measured by enzyme-linked immunosorbent assay (ELISA). One of the greatest difficulties we faced in developing the ELISA protocol was finding a way to attach the peptides to the microplate well surface. Because of their small sizes, the efficiency of coating the exorphin peptides by standard methods (such as those in Aim 1) was extremely low. The approaches we took ranged from drying the peptides down in an ethanol solution to synthesizing biotinylated peptides and bringing them into contact with streptavidin-coated wells. Neither of these approaches worked satisfactorily. A procedure, utilizing glutaraldehyde for pre-coating of plates, was developed and used as follows. 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 μ L/well of a 0.01 mg/mL solution of each peptide in 0.1 M carbonate buffer (pH 9.6) or were left uncoated to serve as control wells. After incubation at 37 °C for 1 h, all wells were washed and blocked by incubation with 1% bovine serum albumin (BSA) in phosphate buffered saline containing 0.05% Tween-20 (PBST) for 1.5 h at room temperature. Serum samples were diluted at 1:800, added at 50 μ L/well in duplicates, and incubated for 1 h. After washing the wells, they were incubated with HRP-conjugated sheep anti-human IgG (GE Healthcare, Piscataway, N.J.) secondary antibodies for 50 min. The plates were washed and 50 μ L of developing solution, comprising 27 Mm citric acid, 50 Mm Na₂HPO₄, 5.5 Mm o-phenylenediamine, and 0.01% H₂O₂ (pH 5), was added to each well. After incubating the plates at room temperature for 40 min, absorbance was measured at 450 nm. All samples were tested in duplicates. Absorbance values were corrected for non-specific binding by subtraction of the mean absorbance of the associated BSA-coated wells. The normalization procedure was as described before in Aim 1 tasks.

Children with autism and increased antibody reactivity to gliadin exhibited elevated levels of IgG antibody to A5, B5, and BCM7 when compared with healthy controls ($p < 0.05$). These subjects clearly had greater antibody levels than those without antibody reactivity to gliadin, although the differences did not reach statistical significance due to small sample size. The elevated antibody to the specific exorphins and their association with increased antibody reactivity to gliadin and casein lends support for the presence of the gluten- and casein-derived peptides in circulation. These results are highly intriguing, but they are preliminary and need to be further examined in larger cohorts. Extensive follow-up studies by our group have been planned and we are in the process of requesting funding for them.

KEY RESEARCH ACCOMPLISHMENTS:

- Received stored and de-identified serum specimens of autism patients, unaffected siblings, age-matched unrelated healthy controls, and celiac disease patients from project collaborators.
- Carried out detailed analysis of IgG and IgA antibodies to gluten, casein (α , β , and κ), deamidated gluten, and TG2 enzyme in all of the above serum samples.
- Found a significantly higher IgG antibody response to gluten in patients with autism in comparison to healthy individuals.
- Found that the abnormally elevated immune response to gluten in ASD differs significantly from that in celiac disease, appearing to be independent of the enzymatic activity of TG2 or presentation via HLA-DQ2 or -DQ8 molecules of antigen-presenting cells.
- Found a significant association between elevated anti-gluten antibody level and the presence of GI symptoms in autistic children.
- Found a significantly higher IgG antibody response to casein, particularly β and κ subunits, in patients with autism in comparison to healthy individuals.
- Demonstrated the specificity of the observed antibody response to gluten and casein in children with autism.
- Found an increase in antibody reactivity to specific casein and gluten peptides (BCM7, A5, and B5) that have been shown to have opioid-like properties in several mouse models.

REPORTABLE OUTCOMES:

I. Manuscripts:

- 1) Ajamian A, Kosofsky B, Wormser GP, Rajadhyaksha AM, **Alaedini A**. Serologic markers of Lyme disease in children with autism. JAMA 2013;309:1771-3. PMID: 23632714.
- 2) Lau NM, Green PH, Taylor A, Hellberg D, Ajamian M, Tan CZ, Kosofsky B, Higgins J, Rajadhyaksha A, **Alaedini A**. Markers of celiac disease and gluten sensitivity in children with autism. PLoS ONE 2013;8:e66155. PMID: 23823064.
- 3) Moeller S, Lau NM, Green PH, Hellberg D, Higgins JJ, Rajadhyaksha AM, **Alaedini A**. Lack of association between autism and anti-GM1 ganglioside antibody. Neurology. 2013 [Epub ahead of print] PMID: 24068789.

II. Abstracts/Presentations:

2012 Annual Meeting of the American Neurological Association: Lau NM, Green PH, Hellberg, D, Higgins JJ, Kosofsky BK, Rajadhyaksha AM, Alaedini A. Elevated immune response to gluten in autism: association with gastrointestinal symptoms but not celiac disease. Ann Neurol. 2012;72(Suppl. 16):S31.

III. Media coverage of research findings:

Reuters. Increased immune reactivity to gluten found in some children with autism. August 8, 2013 (<http://newsdaily.cms.newscred.com/article/e5bc1a705e960f257600d1bd0dfcb602/increased-immune-reactivity-to-gluten-found-in-some-children-with-autism>).

Science Daily. Elevated gluten antibodies found in children with autism. July 20, 2013 (<http://www.sciencedaily.com/releases/2013/06/130620132144.htm>).

United Press International. Higher antibodies to gluten proteins in children with autism. June 20, 2013 (http://www.upi.com/Health_News/2013/06/20/Higher-antibodies-to-gluten-proteins-in-children-with-autism/UPI-19351371779138/).

Psychiatric Annals. Children with autism may have elevated immune response to gluten. July 12, 2012 (<http://www.healio.com/psychiatry/autism-spectrum-disorders/news/online/%7B2793be55-073e-4484-8267-4eab9fed62e1%7D/children-with-autism-may-have-elevated-immune-response-to-gluten>).

Los Angeles Times. Lyme disease, autism link cast into doubt. May 1, 2013 (<http://articles.latimes.com/2013/may/01/science/la-sn-sci-lyme-disease-autism-link-20130430>).

IV. Funding applied for based on work supported by this award:

DOD Idea Development Award

Proposed dates: 3/01/2014 to 2/29/2017

Funding agency: Department of Defense

Title: Proteomic Mapping of the Immune Response to Gluten in Children with Autism

CONCLUSIONS:

A subset of children with autism displays elevated immune reactivity to gluten and casein, which is strongly associated with GI symptoms, but not with celiac disease. The mechanism and molecular specificity of the immune response to gluten in autism appear to be distinct from that in celiac disease, being independent of the activity of TG2 and antigen presentation by HLA-DQ2/DQ8 molecules. The results offer novel opportunities for further research to characterize the immune response to gluten and casein, which may be utilized to identify novel biomarkers of autism and gain novel insights into disease mechanism

So what section:

We can consider some possibilities to explain the higher anti-gliadin and anti-casein antibody levels found in children with autism. Previously, associations between autism and increased GI symptoms, as well as impaired intestinal permeability, have been reported [9-11]. Increased intestinal permeability resulting from damage to the intestinal epithelial barrier in those with autism may be responsible for increased exposure of the immune system to partially digested gluten and casein fragments, resulting in the detected increase in antibody response. The observation here that anti-gliadin antibody reactivity is elevated in patients with GI symptoms lends some support for this idea. The increased intestinal permeability may allow access to circulation by potentially harmful molecules, including certain gluten and casein exorphin peptides as shown in our study, as well as microbial translocation that can lead to systemic inflammation, in the affected individuals.

However, the fact that the higher anti-gliadin and anti-casein antibodies in autistic children were limited to the IgG isotype, without a concomitant rise in IgA, may imply a non-mucosal and/or gluten/casein-independent origin for the observed antibody reactivity. One possibility is that the IgG-specific antibody response in children with autism would have been triggered by ingested gluten or casein at some point in the past, but no longer dependent on continuous mucosal exposure to the proteins. Alternatively, the detected antibodies may be unrelated to gluten and casein as the immunogens. Various immune abnormalities have been demonstrated in autistic children, including increased antibody reactivity to autoantigens [12-14]. It is conceivable that certain autism-associated autoantibodies, the exact targets of which are yet to be identified, would cross-react with one or more gluten and casein proteins and contribute to the detected difference in antibody level between patients and controls. Circulation levels of such antigen-independent or gluten/casein cross-reactive antibodies would not be expected to respond to dietary gluten restriction.

Results of this study are intriguing in the context of disease pathophysiology and biomarker identification. The observed increase in antibody reactivity to gliadin and casein in over one fifth of the autism cohort points to potential shared genetic and/or environmental associations in a sizable subset of patients. As such, the generated data provide an impetus to further examine the affected patient subset for additional immunologic and genomic clues. It is possible that, in a subset of children with autism, the condition is associated with antibody reactivity to a unique set of proteins/epitopes that would be significantly different from the pattern of antibody response in celiac disease and other conditions. This specific pattern of antibody reactivity may be useful as a source of biomarkers. A unique antibody response to particular gluten or casein epitopes could also be associated with specific HLA genes in that disease subset.

In conclusion, the increased anti-gliadin and anti-casein antibody responses in autism points to a potential mechanism involving immunologic and/or intestinal permeability abnormalities in a subset of patients. The observed antibody reactivity to gliadin in most children with autism appears to be unrelated to celiac disease. Therefore, the heightened immune response to gluten in autism deserves further attention and research in determining its utility as a source of biomarkers and clues regarding

disease pathophysiology. Better understanding of this immune response may offer novel markers for the identification of subsets of patients who would be responsive to specific treatment strategies.

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Markers of Celiac Disease and Gluten Sensitivity in Children with Autism

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Abstract

Objective: Gastrointestinal symptoms are a common feature in children with autism, drawing attention to a potential association with celiac disease or gluten sensitivity. However, studies to date regarding the immune response to gluten in autism and its association with celiac disease have been inconsistent. The aim of this study was to assess immune reactivity to gluten in pediatric patients diagnosed with autism according to strict criteria and to evaluate the potential link between autism and celiac disease.

Methods: Study participants included children (with or without gastrointestinal symptoms) diagnosed with autism according to both the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview, Revised (ADI-R) ($n=37$), their unaffected siblings ($n=27$), and age-matched healthy controls ($n=76$). Serum specimens were tested for antibodies to native gliadin, deamidated gliadin, and transglutaminase 2 (TG2). Affected children were genotyped for celiac disease associated HLA-DQ2 and -DQ8 alleles.

Results: Children with autism had significantly higher levels of IgG antibody to gliadin compared with unrelated healthy controls ($p<0.01$). The IgG levels were also higher compared to the unaffected siblings, but did not reach statistical significance. The IgG anti-gliadin antibody response was significantly greater in the autistic children with gastrointestinal symptoms in comparison to those without them ($p<0.01$). There was no difference in IgA response to gliadin across groups. The levels of celiac disease-specific serologic markers, i.e., antibodies to deamidated gliadin and TG2, did not differ between patients and controls. An association between increased anti-gliadin antibody and presence of HLA-DQ2 and/or -DQ8 was not observed.

Conclusions: A subset of children with autism displays increased immune reactivity to gluten, the mechanism of which appears to be distinct from that in celiac disease. The increased anti-gliadin antibody response and its association with GI symptoms points to a potential mechanism involving immunologic and/or intestinal permeability abnormalities in affected children.

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Introduction

Glutens are the major storage proteins of wheat and related cereals, comprising over 70 different molecules in any given wheat variety [1]. The main classes of gluten include α/β -gliadins, γ -gliadins, ω -gliadins, high molecular weight glutenins, and low molecular weight glutenins [2]. Gluten sensitivity can be defined as a state of heightened immunologic reaction to gluten proteins, which may be accompanied by increased levels of antibodies against them. Heightened immune reactivity to gluten is recognized and understood best in the context of celiac disease, an autoimmune disorder primarily targeting the small intestine, and wheat allergy [3]. The humoral immune response in celiac

disease also includes antibodies to deamidated sequences of gliadin and to the autoantigen transglutaminase 2 (TG2), which are highly specific and sensitive serologic markers of the condition [4]. Celiac disease is also closely linked with genes that code for human leukocyte antigens (HLA) DQ2 and DQ8 [5].

While the etiology and pathogenesis of autism are poorly understood, there is evidence that immune system abnormalities are associated with symptoms in a substantial number of affected individuals [6]. In addition, several studies have evaluated gastrointestinal (GI) symptoms and defects in GI barrier function in patients with autism [7–10]. A possible association between autism and celiac disease was first discussed over 40 years ago [11,12]. Although some studies have pointed to higher frequency

of celiac disease, family history of celiac disease, or elevated antibody to gliadin among autistic children [13–15], others have not supported these findings [16–18]. Diets that exclude gluten are becoming increasingly popular in the autism community, but their effectiveness has not been proven in controlled and blinded studies [19]. Despite years of speculation and immense interest by families of affected children regarding the potential connection between autism and gluten sensitivity, no well-controlled study has been performed to determine the levels of immune reactivity to gluten in patients, to characterize the antigenic specificity of this immune response, or to assess its pathogenic relevance to autism. In this study, we examine and compare markers of celiac disease and gluten sensitivity in cohorts of individuals diagnosed with autism, unaffected siblings of the patients with autism, and unrelated healthy controls.

Methods

Patients and Controls

The study included 140 children, including 37 with autism, 27 unaffected siblings of similar ages within the same families, and 76 unrelated healthy controls. Serum samples from individuals with autism and their siblings were acquired from the Autism Genetic Resource Exchange (AGRE). DNA samples from the 37 children with autism were also provided by AGRE. Participants in the AGRE program have been recruited primarily from the north-eastern and western United States. Affected children met the diagnostic criteria for autism based on both the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview, Revised (ADI-R). All available serum samples satisfying the above criteria were included. Information on GI symptoms was based on parent questionnaires, interviews, and medical histories. The data collected by AGRE from these evaluations were retrieved from the online AGRE phenotype database. The control sera were from healthy children in the United States ($n = 14$) and Sweden ($n = 62$). The healthy controls from U.S. resided primarily in Connecticut, north New Jersey, and New York City, and were recruited in a general pediatric clinic at the Weill Cornell Medical College. The healthy controls from Sweden were recruited at child health care centres and schools in the Falun region of central Sweden [20]. Screening questionnaires were used to evaluate the general health of the U.S. and Swedish controls, and individuals who reported having a chronic disease were not included. Serum from a biopsy-proven celiac disease patient, diagnosed according to previously described criteria [21] at Columbia University Medical Center, was used as a positive control for the antibody assays. Written informed consent was obtained for all study participants from the individual, next of kin, caretaker, or guardian. The consent procedures were approved by the Institutional Review Boards of the involved organizations (AGRE, Columbia University, Weill Cornell Medical College, and Uppsala University). Complete documentation of consent is maintained at the respective organizations. This specific study was approved by the Institutional Review Board of Columbia University Medical Center. Specimens were kept at -80°C to maintain stability.

Gliadin

The antigen mixture used for the anti-gliadin antibody assays was the Prolamine Working Group (PWG) reference gliadin, which was extracted from a combination of 28 different wheat varieties, as previously described [22]. The protein profile of the PWG gliadin extract was assessed by SDS-polyacrylamide gel electrophoresis, using 10% NuPAGE Bis-Tris precast gels and 3-

(N-morpholino)propanesulfonic acid (MOPS) buffer (Life Technologies, Carlsbad, Calif.).

Anti-gliadin Antibodies

Serum IgG and IgA antibodies to gliadin were measured separately by enzyme-linked immunosorbent assay (ELISA) as previously described [23,24], with some modifications. A 2 mg/mL stock solution of the PWG gliadin was prepared in 60% ethanol. 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 μL /well of a 0.01 mg/mL solution of PWG gliadin in 0.1 M carbonate buffer (pH 9.6) or were left uncoated to serve as control wells. After incubation at 37°C for 1 h, all wells were washed and blocked by incubation with 1% bovine serum albumin (BSA) in phosphate buffered saline containing 0.05% Tween-20 (PBST) for 1.5 h at room temperature. Serum samples were diluted at 1:800 for IgG measurement and at 1:200 for IgA measurement, added at 50 μL /well in duplicates, and incubated for 1 h. Each plate contained a positive control sample from a patient with biopsy-proven celiac disease and elevated IgG and IgA antibodies to gliadin. After washing the wells, they were incubated with HRP-conjugated anti-human IgG (GE Healthcare, Piscataway, N.J.) or IgA (MP Biomedicals, Santa Ana, Calif.) secondary antibodies for 50 min. The plates were washed and 50 μL of developing solution, comprising of 27 mM citric acid, 50 mM Na_2HPO_4 , 5.5 mM α -phenylenediamine, and 0.01% H_2O_2 (pH 5), was added to each well. After incubating the plates at room temperature for 20 min, absorbance was measured at 450 nm. All serum samples were tested in duplicate. Absorbance values were corrected for non-specific binding by subtraction of the mean absorbance of the associated BSA-coated wells. The corrected values were first normalized according to the mean value of the positive control duplicate on each plate. The mean antibody level for the unrelated healthy control cohort was then set as 1.0 AU and all other results were normalized accordingly.

Anti-transglutaminase 2 (TG2) Antibodies

IgA antibody to recombinant human TG2 was measured in sera using an ELISA kit, according to the manufacturer's protocol (Euroimmun, Lubeck, Germany).

Anti-deamidated Gliadin Antibodies

Sera were tested separately for IgG and IgA antibodies to a previously described glutamine-glutamate substituted trimer of a fusion peptide containing the sequences PLQPEQFPF and PEQLPQFEE [25] by ELISA, according to the manufacturer's protocols (Euroimmun).

HLA Typing

High resolution HLA genotyping was performed by multiplex polymerase chain reaction (PCR) with biotinylated primers, followed by reverse hybridization of the PCR products to line arrays of sequence-specific DQA1 and DQB1 oligonucleotide probes, using INNO-LiPA HLA-DQ kits, according to the manufacturer's instructions (Innogenetics, Gent, Belgium). Presence or absence of celiac disease-associated DQA1*0501/0505-DQB1*0201/0202 (DQ2) and DQA1*03-DQB1*0302 (DQ8) genes was determined.

Data Analysis

Differences between groups were analyzed by the two-tailed Student's *t* test, Welch's *t* test, Mann-Whitney U test, or one-way analysis of variance (ANOVA) with post-hoc Dunn test (continuous data), and the Fisher's exact test (nominal data). Adjustment

for covariate effect (age, gender, and race) was carried out by analysis of covariance (ANCOVA), using the general linear model. Logistic regression was used to calculate the odds ratios associated with increased antibodies in individuals with autism. For these analyses, increased levels of anti-gliadin antibody were defined as values at the 95th percentile or higher in the unrelated healthy control group. For IgA anti-TG2 antibody and IgG/IgA anti-deamidated gliadin antibodies, cutoffs for positivity were assigned by the manufacturer. Differences with p values of <0.05 were considered to be statistically significant. Statistical analyses were performed with Prism 5 (GraphPad, San Diego, Calif.) and Minitab 16 (Minitab, State College, Pa.).

Results

Patients and Controls

The demographic and clinical characteristics of the patients with autism, their unaffected siblings, and unrelated healthy controls are shown in Table 1. The patient cohort included four individuals on gluten-free diet. Because the effect of gluten-free diet on antibody levels in autism is not known, these patients were not excluded from the study.

Gliadin

The gel electrophoresis profile for the PWG gliadin used in anti-gliadin antibody assays indicated the presence of all main types of gliadin proteins, α/β , γ , and ω . The mixture also contained high and low molecular weight glutenin subunits (Fig. 1).

Antibody Levels

Mean levels of IgG and IgA class antibodies to gliadin in patient and control groups are presented in Fig. 2. Children with autism exhibited significantly elevated levels of IgG antibody to gliadin when compared with unrelated healthy controls or when compared with the combination of unaffected siblings and unrelated healthy controls ($p<0.01$). The difference remained significant after adjusting for the covariates of age, gender, and race ($p<0.01$). The anti-gliadin IgG differences between the children with autism and their unaffected siblings, and between the siblings and unrelated healthy controls, did not reach statistical significance. Based on the stated cutoff for positivity (95th percentile of the healthy control group), 8/33 (24.2%) of the children with autism, excluding those who reported being on gluten-free diet, 8/37 (21.6%) of all autistic children, including those on gluten-free diet, 2/27 (7.4%) of unaffected siblings, and 4/76 (5.3%) of unrelated healthy children were positive for IgG anti-gliadin antibody, indicating a significantly higher frequency in those with autism compared to unrelated healthy controls ($p<0.01$). Children with autism had increased odds of having elevated IgG antibody to gliadin in comparison to healthy controls (odds ratio: 4.97; 95% confidence interval: 1.39–17.8). The

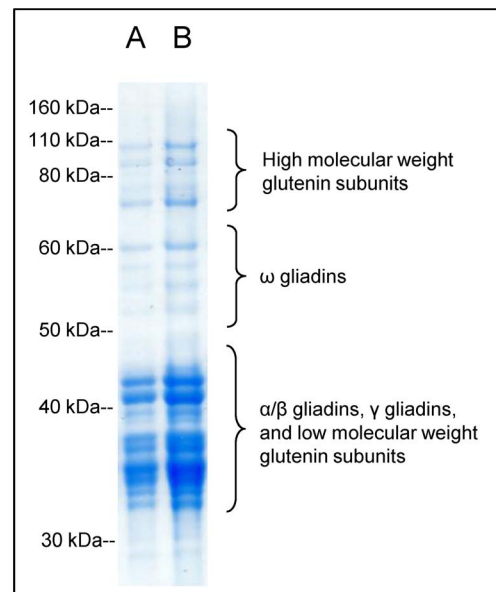


Figure 1. Gel electrophoresis profile of the PWG gliadin preparation used for the anti-gliadin antibody assays. A) 5 µg of protein loaded; B) 10 µg of protein loaded.
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differences in levels of IgA antibody to gliadin among the three groups were not significant.

All patients and controls were also tested for the currently recommended full panel of the most sensitive and specific serologic markers of celiac disease, including IgA antibody to TG2, IgG antibody to deamidated gliadin, and IgA antibody to deamidated gliadin. None of the individuals in any group were positive for IgA antibody to TG2. Two of 37 autistic children, 3 of 27 unaffected siblings, and none of 76 unrelated healthy controls had values above the manufacturer's assigned cutoff for IgG antibody to deamidated gliadin. Similarly, none of 37 autistic children, 1 of 27 unaffected siblings, and 1 of 76 unrelated healthy controls were positive for IgA antibody to deamidated gliadin.

All four individuals who were on gluten-free diet were negative for anti-gliadin, anti-deamidated gliadin, and anti-TG2 antibodies.

HLA Typing

In the group of children with autism, 18/37 (48.6%) were positive for HLA-DQ2 and/or -DQ8 (6 DQ2, 12 DQ8). There was no clear association between antibody to gliadin and the presence of celiac disease-associated HLA-DQ2/DQ8 in patients with autism: 3/8 (37.5%) of the anti-gliadin antibody-positive individuals with autism displayed HLA-DQ2 and/or DQ8 (2

Table 1. Demographic characteristics of study cohorts.

Subject group	Number of subjects	Mean age— years $\pm$ SD	Male sex— no. (%)	White race no. (%)
Autism	37	7.8 $\pm$ 2.9	29 (78)	33 (89)
With GI symptoms	19	7.1 $\pm$ 2.3	13 (68)	15 (79)
Without GI symptoms	8	7.1 $\pm$ 2.3	6 (75)	8 (100)
Unaffected sibling	27	8.1 $\pm$ 2.9	18 (67)	25 (93)
Unrelated healthy	76	8.8 $\pm$ 3.7	59 (77)	70 (92)

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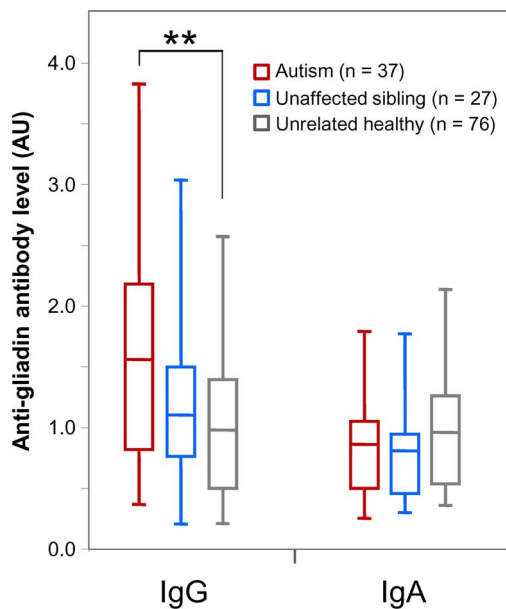


Figure 2. Comparison of levels of IgG and IgA antibody to gliadin in children with autism, their unaffected siblings, and unrelated healthy controls. Boxed segments represent the middle 50% of the data. Whiskers indicate the range of data. Large horizontal bars indicate mean value of the data. **= $p<0.01$. doi:10.1371/journal.pone.0066155.g002

DQ2, 1 DQ8), while 15/29 (51.7%) of those below the cutoff for antibody positivity had DQ2 and/or DQ8. Neither of the two patients with autism who were positive for IgG anti-deamidated gliadin antibody had DQ2 or DQ8. About 95% or more of celiac disease patients carry HLA-DQ2 and/or -DQ8, compared to an estimated 40% of the U.S. general population [26].

GI Symptoms

Medical histories were available for 27 of the 37 children with autism. 19/27 (70.3%) reported persistent GI symptoms, including 10 with chronic loose stools or diarrhea, 2 with gastroesophageal reflux, 3 with frequent stools, 3 with constipation, and 1 with non-specified GI symptoms. Affected patients with GI symptoms were found to have significantly higher levels of IgG antibody to gliadin when compared to patients without GI symptoms ($p<0.01$) (Fig. 3A). This difference remained significant after adjusting for the covariates of age, gender, and race ($p<0.01$). Information on GI symptoms was available for 5 of the 8 children whose anti-gliadin antibody levels were determined to be above the cutoff. They included 3 with chronic loose stools or diarrhea, 1 with frequent stools, and 1 with constipation.

There was no significant difference in the levels of IgA antibody to gliadin (Fig. 3A), IgG and IgA antibodies to deamidated gliadin (Fig. 3B), and IgA antibody to TG2 (Fig. 3C) between patients with GI complaints and those without. One autism patient with GI symptoms was positive for IgG antibody to deamidated gliadin, while the remaining patients in both groups were negative for all other markers.

Discussion

The aim of this study was to carry out a comprehensive analysis of markers of celiac disease and gluten sensitivity in a group of children with autism who had been diagnosed according to strict criteria and defined instruments. Our data indicate that children

with autism have higher levels of IgG antibody to gliadin compared to healthy controls. In addition, among patients with autism, the antibody response to gliadin was greater in those with GI symptoms. However, in contrast to patients with celiac disease, no association was observed between the elevated anti-gliadin antibody level and the presence of highly specific serologic markers of celiac disease or HLA-DQ2/DQ8. The findings indicate that the observed anti-gliadin immune response in patients with autism is likely to involve a mechanism that is distinct from celiac disease, without the requirement for TG2 activity or antigen presentation through DQ2/DQ8 MHC molecules [27].

The data from this study should be interpreted with caution. Most importantly, the observed increased IgG antibody response to gliadin does not necessarily indicate sensitivity to gluten or any pathogenic role for antibodies to gliadin in the context of autism. In addition, the results do not rule out the possibility of moderately increased prevalence of celiac disease among children with autism, especially as duodenal biopsy, the gold standard for definitive diagnosis of celiac disease, was not performed. However, considering the excellent sensitivity and specificity of anti-TG2 and (and to a lesser extent anti-deamidated gliadin) antibodies, as well as the high negative predictive value of HLA-DQ2/DQ8 markers for celiac disease, it can be concluded with high certainty that the overwhelming majority of autism patients with elevated antibody to gliadin do not have celiac disease. If future studies prove the existence of sensitivity to gluten in a subset of patients with autism, the gluten-associated symptoms in such individuals may fall within the spectrum of “non-celiac gluten sensitivity” [28].

Compared to previous reports examining the link between celiac disease/gluten sensitivity and autism, this study is unique in several ways. First, a shortcoming in earlier studies has been the lack or incompleteness of suitable age-matched healthy control groups necessary for this type of analysis. In this work, the antibody levels in children with autism were compared to two separate pediatric control groups: unaffected siblings of the same patients, as well as a larger cohort of unrelated healthy children. Second, previous reports have used specimens from more heterogeneous groups of patients generally recruited at local hospitals or clinics, and while most report the use of DSM diagnostic criteria, it is unclear which test(s) informed the final diagnosis of autism. In contrast, the samples in this study were acquired from a well-recognized repository of biomaterials (AGRE), which is managed by the world's largest autism advocacy organization and has been utilized in various past research projects. The associated AGRE database includes information about family pedigree, scores from various tests and questionnaires, and medical histories for many of the patients for which biospecimens are available. Patients in this study were selected only if they were identified as having autism according to two separate instruments, ADOS and ADI-R, thus greatly increasing the likelihood of accurate diagnosis.

A limitation of this study is that we could not control for geographical distribution, socioeconomic status, or diet of the research participants. These factors may contribute to levels of antibodies against dietary and other antigens in patients and controls. In addition, information on GI symptoms was available only for some patients and none of the controls. Access to such data would have strengthened the study's finding regarding the association between GI symptoms and anti-gliadin antibody levels. As such, the conclusions of this study should be considered preliminary, requiring further confirmation in larger and better-characterized cohorts of patients and controls.

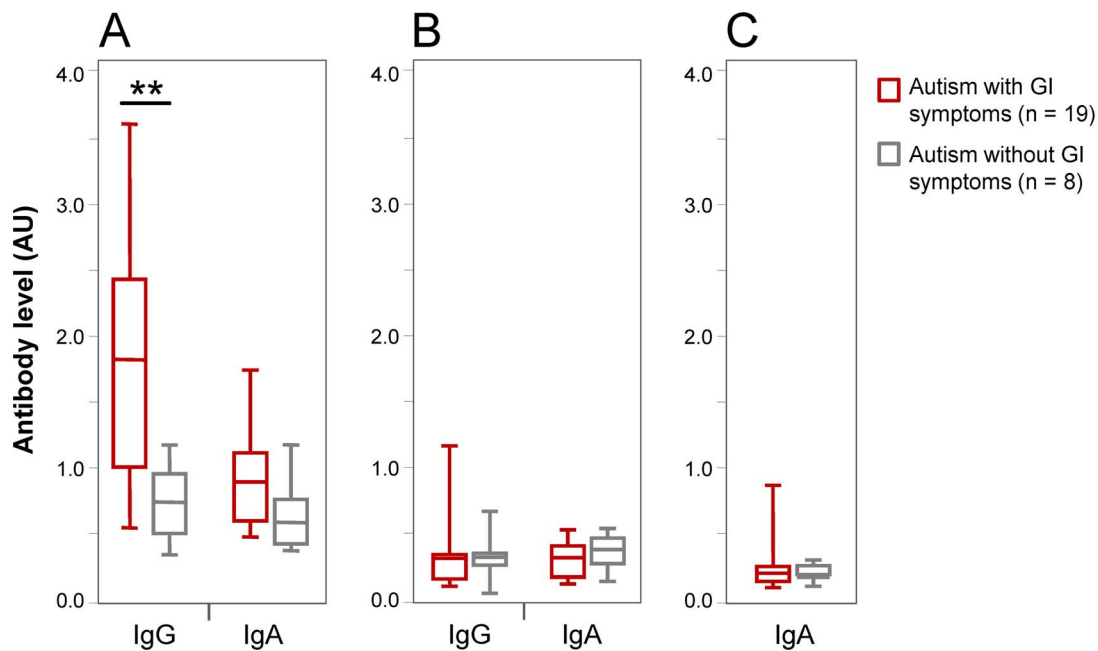


Figure 3. Comparison of levels of antibody to A) gliadin, B) deamidated gliadin fusion peptide, and C) human TG2 in autistic children, with and without GI symptoms. Boxed segments represent the middle 50% of the data. Whiskers indicate the range of data. Large horizontal bars indicate mean value of the data. $** = p < 0.01$. doi:10.1371/journal.pone.0066155.g003

We can consider some possibilities to explain the higher anti-gliadin antibody levels found in the cohort of children with autism. Previously, associations between autism and increased GI symptoms, as well as impaired intestinal permeability, have been reported [9,10,29]. Increased intestinal permeability resulting from damage to the intestinal epithelial barrier in those with autism may be responsible for increased exposure of the immune system to partially digested gluten fragments, resulting in the detected increase in antibody response. The observation here that anti-gliadin antibody reactivity is elevated in patients with GI symptoms lends some support for this idea. At the same time, the fact that the higher anti-gliadin antibodies in autistic children were limited to the IgG isotype, without a concomitant rise in IgA, may imply a non-mucosal and/or gluten-independent origin for the observed antibody reactivity. One possibility is that the IgG-specific antibody response in children with autism would have been triggered by ingested gluten at some point in the past, but no longer dependent on continuous mucosal exposure to the proteins. Alternatively, the detected anti-gliadin antibodies may be unrelated to gluten as the immunogen. Various immune abnormalities have been demonstrated in autistic children, including increased antibody reactivity to autoantigens [30–32]. It is conceivable that certain autism-associated autoantibodies, the exact targets of which are yet to be identified, would cross-react with one or more gluten proteins and contribute to the detected difference in anti-gliadin antibody level between patients and controls. Circulation levels of such antigen-independent or gluten cross-reactive antibodies would not be expected to respond to dietary gluten restriction.

Results of this study are intriguing in the context of disease pathophysiology and biomarker identification. The observed increase in antibody reactivity to gliadin in over one fifth of the autism cohort points to potential shared genetic and/or environmental associations in a sizable subset of patients. As such, the generated data provide an impetus to further examine the affected patient subset for additional immunologic and genomic clues. It is possible that, in a subset of children with autism, the condition is

associated with antibody reactivity to a unique set of gluten proteins that would be significantly different from the pattern of anti-gliadin antibody response in celiac disease and other conditions. This specific pattern of antibody reactivity may be useful as a source of biomarkers. A unique antibody response to particular gluten molecules could also be associated with specific HLA genes in that disease subset.

In conclusion, the increased anti-gliadin antibody response in autism and its association with GI symptoms points to a potential mechanism involving immunologic and/or intestinal permeability abnormalities in a subset of patients. The observed antibody reactivity to gliadin in most children with autism appears to be unrelated to celiac disease. Therefore, the heightened immune response to gluten in autism deserves further attention and research in determining its utility as a source of biomarkers and clues regarding disease pathophysiology. Better understanding of this immune response may offer novel markers for the identification of subsets of patients who would be responsive to specific treatment strategies.

Acknowledgments

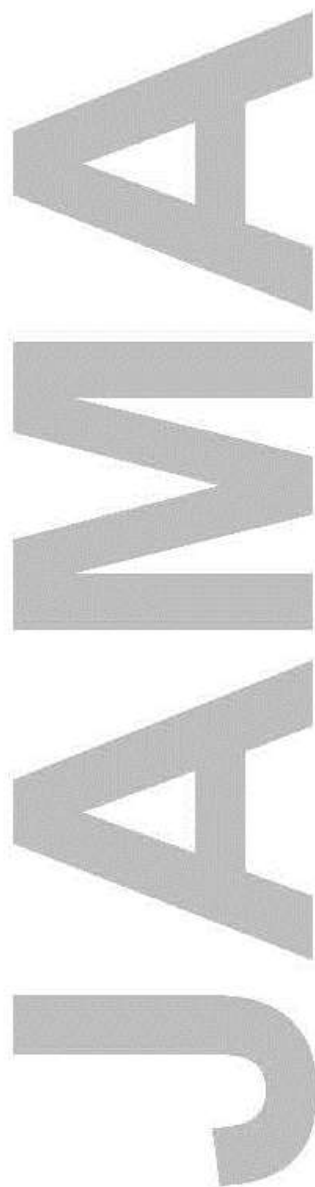
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Author Contributions

Conceived and designed the experiments: AA AMR. Performed the experiments: NML AKT MA CZT. Analyzed the data: NML AKT AA. Contributed reagents/materials/analysis tools: PHG DH AMR JJH BEK. Wrote the paper: AA NML AMR PHG AKT MA BEK DH JJH CZT.

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RESEARCH LETTER

Serologic Markers of Lyme Disease in Children With Autism

To the Editor: A proposed link between Lyme disease and autism has garnered considerable attention.^{1,2} Among individuals with autism spectrum disorders, rates of seropositivity for Lyme disease of greater than 20% have been reported.¹ However, controlled studies to assess serological evidence of infection with *Borrelia burgdorferi* (the causative agent of Lyme disease) in patients with autism are lacking.

Serological evidence of infection with *B burgdorferi* is essential for diagnosing Lyme disease, except in cases of

typical erythema migrans skin lesions. To evaluate the suggestion that autism is commonly linked to Lyme disease, we performed Lyme disease serological testing on serum samples from children with autism and those without autism.

Methods. Serum samples from 120 children aged 2 through 18 years with autism and those without autism were acquired from the Autism Genetic Resource Exchange (AGRE) (37 with autism and 27 unaffected siblings) and the Weill Cornell Autism Research Program (WCARP) (33 with autism, 8 unaffected siblings, and 15 unrelated healthy controls). All WCARP and some unselected AGRE sites collected serum samples; all available serum samples were included.

Patients from the AGRE program met diagnostic criteria for autism based on both the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview, Revised, whereas WCARP patients met criteria for autism based only on the ADOS. Participants in the AGRE program have been recruited primarily from the northeastern and western United States; serum samples for this study were collected from August 31, 1999, through April 25, 2001.

The WCARP serum samples were from participants who resided primarily in Connecticut, New Jersey, and New York, and were collected from May 19, 2010, through March 7, 2012. Screening questionnaires were used to evaluate the general health of unrelated controls.

Written informed consent was obtained for all study participants from a parent or guardian. Serum samples from 2 patients with culture-confirmed early Lyme disease were used as positive controls. Specimens were kept at -80°C to maintain stability. This study was approved by the institutional review board of Columbia University Medical Center.

Testing for antibodies to *B burgdorferi* was performed according to the 2-tier algorithm recommended by the US Centers for Disease Control and Prevention.³ Initial screening for anti-*B burgdorferi* immunoglobulin G and M antibodies was performed with separate enzyme-linked immunosorbent assays (ELISAs), according to the manufacturer's protocols (Euroimmun). Specimens classified as borderline or positive were further tested by Western blotting for IgG or IgM antibodies to electrophoresis-separated *B burgdorferi* strain B31 proteins (Euroimmun).⁴

Assuming 1% or lower seroprevalence in controls, and at least 20% seroprevalence in cases as suggested, the sample size in this study would provide greater than 90% power with an α level of .05. Differences between groups were analyzed using the 2-tailed Fisher exact test; *P* values of less than .05 were considered to be statistically significant. Binomial distribution confidence intervals were determined by the Clopper-Pearson exact method.

Results. Seventy children with autism (58 male; mean [SD] age, 7.2 [3.6] years) and 50 unaffected controls (32 male; mean age, 9.0 [4.0] years) were included. Of the patients with autism, 1 was positive by ELISA for anti-*B burgdorferi* IgG, whereas 4 were borderline by ELISA for IgM. Of the 50 children in the unaffected control group, 4 were positive and 1 was borderline for IgG by ELISA, whereas 1 was positive by ELISA for IgM.

All serum samples that were positive or borderline by ELISA were further analyzed using Western blot and were found to be negative for anti-*B burgdorferi* antibody reactivity (TABLE 1 and TABLE 2). The 95% confidence interval for seroprevalence in children with autism and in unaffected controls was 0% to 5.1%.

Discussion. None of the children with autism or unaffected controls had serological evidence of Lyme disease by 2-tier testing. A potential limitation of this study is the lack of information about lifestyle for patients and controls, including time spent outdoors.

The data do not address whether Lyme disease may cause autism-like behavioral deficits in some cases. However, the study's sample size is large enough to effectively rule out the suggested high rates of Lyme disease or associated seroprevalence among affected children.

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Table 1. Serum Immunoglobulin G Antibody Reactivity to *Borrelia burgdorferi* Protein Bands as Determined by Western Blotting in Patients and Controls Who Were Positive or Borderline for IgG by Enzyme-Linked Immunosorbent Assay^a

Serum Sample No.	Group	Western Blot Band ^b											
		p18	p25	p28	p30	p31	p34	p39	p41	p45	p58	p66	p93
1	Autism ^c								+			+	
2	Unaffected control ^c				+				+				
3	Unaffected control ^c								+				
4	Unaffected control ^c								+		+		
5	Unaffected control ^c	+							+			+	+
6	Unaffected control ^c								+				
12	Lyme disease control ^d	+	+					+	+	+		+	
13	Lyme disease control ^c		+						+	+			

^aNone of the children with autism or unaffected controls had serological evidence of Lyme disease by 2-tier testing. The 95% confidence interval for IgG seroprevalence in children with autism and in unaffected controls was 0% to 5.1%.

^bAccording to Centers for Disease Control and Prevention testing criteria, an IgG immunoblot was considered positive if 5 or more of the 10 following protein bands reacted positively: p18, p25 (OspC), p28, p30, p39 (BmpA), p41 (FlaB), p45, p58, p66, and p93.³

^cIndividual did not meet IgG seropositivity criteria for Lyme disease.

^dIndividual met IgG seropositivity criteria for Lyme disease.

Table 2. Serum Immunoglobulin M Antibody Reactivity to *Borrelia burgdorferi* Protein Bands as Determined by Western Blotting in Patients and Controls Who Were Positive or Borderline for IgM by Enzyme-Linked Immunosorbent Assay^a

Serum Sample No.	Group	Western Blot Band ^b		
		p25	p39	p41
7	Autism ^c			+
8	Autism ^c			+
9	Autism ^c			+
10	Autism ^c			
11	Unaffected control ^c			
12	Lyme disease control ^d	+		+
13	Lyme disease control ^d	+	+	+

^aNone of the children with autism or unaffected controls had serological evidence of Lyme disease by 2-tier testing. The 95% confidence interval for IgM seroprevalence in children with autism and in unaffected controls was 0% to 5.1%.

^bAccording to Centers for Disease Control and Prevention testing criteria, an IgM immunoblot was considered positive if 2 of the 3 following protein bands reacted positively: p25 (OspC), p39 (Bmp A), and p41 (FlaB).³

^cIndividual did not meet IgM seropositivity criteria for Lyme disease.

^dIndividual met IgM seropositivity criteria for Lyme disease.

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Author Contributions: Dr Alaedini had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Alaedini.

Acquisition of data: Ajamian.

Analysis and interpretation of data: Ajamian, Kosofsky, Wormser, Rajadhyaksha, Alaedini.

Drafting of the manuscript: Ajamian, Alaedini.

Critical revision of the manuscript for important intellectual content: Ajamian, Kosofsky, Wormser, Rajadhyaksha, Alaedini.

Statistical analysis: Ajamian, Alaedini.

Obtained funding: Alaedini.

Administrative, technical, or material support: Ajamian, Kosofsky, Wormser, Rajadhyaksha, Alaedini.

Study supervision: Alaedini.

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Clinical/Scientific Notes

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LACK OF ASSOCIATION BETWEEN AUTISM AND ANTI-GM1 GANGLIOSIDE ANTIBODY

Forty of 54 children with autism were reported to have an elevated antibody response to GM1 ganglioside that correlated with disease severity.¹ Anti-ganglioside autoantibodies, especially those directed at GM1, are known to be associated with and play a pathogenic role in some immune-mediated peripheral neuropathies.^{2,3} The presumed link between autism and anti-GM1 antibodies, therefore, implies that testing may identify a sizable subset of patients who would benefit from immunomodulatory therapy. To evaluate the proposed association between autism and anti-GM1 antibodies, we performed serologic testing on children diagnosed with autism by strict clinical criteria and those without autism, using a standard and validated immunoassay protocol.

Methods. Study population. Serum samples were from 181 children, including 70 with autism, 35 unaffected siblings of similar ages within the families of autistic children, and 76 age-matched unrelated healthy controls (table). Samples were obtained from the Autism Genetic Resource Exchange (AGRE) (37 autism and 27 unaffected siblings), the Weill Cornell Autism Research Program (WCARP) (33 autism, 8 unaffected siblings, and 14 unrelated healthy controls), and Uppsala University (62 unrelated healthy controls). AGRE patients met diagnostic criteria for autism based on both the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview, Revised (ADI-R), whereas WCARP patients met criteria for autism based on the ADOS. All available serum samples were included. Serum from a patient diagnosed with multifocal motor neuropathy (MMN) with elevated immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies to GM1 was used as positive control. Specimens were kept at -80°C to maintain stability.

Standard protocol approvals, registrations, and patient consents. Written informed consent was obtained for all study participants. The consent procedures were approved by the institutional review boards of the involved organizations. This specific study was approved by the institutional review board of Columbia University Medical Center.

Measurement of anti-GM1 ganglioside antibodies. IgG and IgM antibodies to GM1 were measured by ELISA as previously described,⁴ with minor modifications. Ninety-six-well round-bottom polystyrene plates (Nunc, Penfield, NY) were coated with $0.5\text{ }\mu\text{g/well}$ of GM1 ganglioside (Sigma-Aldrich, St. Louis, MO) in methanol. Wells that were not coated with antigen served as controls. After methanol evaporation, wells were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 2 hours at 4°C . Sera were diluted serially in blocking buffer, starting at 1:100 for IgG and at 1:400 for IgM measurements. Wells were filled with $50\text{ }\mu\text{L}$ of diluted sera in duplicate (in both antigen-coated and noncoated wells), incubated overnight at 4°C , and then washed with PBS. Each plate contained the MMN positive control sample. Wells were incubated with $50\text{ }\mu\text{L}$ of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Amersham Biosciences, Amersham, UK), at 1:2,000, or HRP-conjugated goat anti-human IgM (ICN Biomedicals, Irvine, CA), at 1:1,000 (2 hours at 4°C). Wells were washed, followed by the addition of $50\text{ }\mu\text{L}$ of developing solution.⁴ Plates were incubated at room temperature for 30 minutes before measuring optical density (OD) at 450 nm. The OD values of antigen-coated wells for each sample were corrected for nonspecific binding by subtracting the OD of the associated noncoated wells. Data were normalized according to the mean value of the positive control duplicate on each plate. The titer for each sample was assigned as the highest dilution at which the OD reading was 0.1 unit greater than in the corresponding control wells.

Data analysis. The differences between groups were analyzed by the 2-tailed Fisher exact test; p values less than 0.05 were considered to be statistically significant. Binomial distribution confidence intervals (CIs) were determined by the Clopper-Pearson exact method.

Results. One autistic child and one pediatric healthy control were found to have a titer of 100 for IgG anti-GM1 antibody. All other sera were negative at the lowest titers examined for IgG and IgM antibodies to GM1. No differences in the frequency of seropositivity for anti-GM1 antibodies were found between patients and controls. The 95% CIs for the rate of

Table Demographic characteristics and experimental data for the study cohorts						
Group	No. of individuals	Mean $\pm$ SD age, y	Male sex, n (%)	White race, n (%)	Anti-GM1 IgG ^a	Anti-GM1 IgM ^a
Autism	70	7.2 $\pm$ 3.6	58 (83)	44 (63)	A: 0.095 $\pm$ 0.035	A: 0.053 $\pm$ 0.008
					C: 0.110 $\pm$ 0.027	C: 0.052 $\pm$ 0.014
Unaffected sibling	35	8.9 $\pm$ 3.9	23 (66)	29 (83)	A: 0.097 $\pm$ 0.036	A: 0.051 $\pm$ 0.008
					C: 0.118 $\pm$ 0.040	C: 0.054 $\pm$ 0.016
Unrelated healthy	76	8.8 $\pm$ 3.7	59 (77)	70 (92)	A: 0.085 $\pm$ 0.027	A: 0.052 $\pm$ 0.008
					C: 0.100 $\pm$ 0.032	C: 0.051 $\pm$ 0.009

Abbreviations: A = antigen-coated wells; C = control noncoated wells; IgG = immunoglobulin G; IgM = immunoglobulin M; OD = optical density.

^a As determined by ELISA. Values indicate mean normalized OD $\pm$ SD. Serum dilutions were at 1:100 for IgG and at 1:400 for IgM measurements.

positivity in children with autism and in unaffected controls were 0% to 7.7% for IgG and 0% to 5.1% for IgM anti-GM1 antibodies.

Discussion. The assay methodology in the present study was based on the widely accepted standard ELISA procedure for the detection of IgG and IgM anti-ganglioside antibodies, which has been utilized and validated in numerous studies by us and other groups.⁴⁻⁷ In addition, all patients in this report were identified as having autism based on detailed neuropsychological testing using either the ADOS or both the ADOS and the ADI-R. The results of this case-control study provide strong evidence against association of autism with increased antibody reactivity to GM1 ganglioside.

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