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Gluten and non-gluten proteins of wheat as target antigens in autism, Crohn's and celiac disease

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ABSTRACT

Studies show that patients with celiac disease react not only with gluten wheat proteins but also with non-gluten wheat components. Our goal was to measure IgG or IgA antibodies against wheat proteins or peptides that would provide the most sensitive method for the detection of wheat immune reaction in children with autism spectrum disorder, and patients with Crohn's and celiac disease (CD). Using ELISA, we measured these antibodies against various gluten and non-gluten wheat proteins. Compared to controls in all three conditions, the strongest reaction was against CXCR3-binding gliadin peptide, followed by a wheat protein mixture, and α-gliadin 33-mer peptide. We determined that a sample that strongly reacted against non-gluten proteins also reacted strongly against gluten proteins. We also found that IgA antibodies against CXCR3-binding gliadin peptide were strongly reactive in a subgroup of 33% in the autism group, 42% in the Crohn's group, and all tested samples with CD. The results indicate that measuring IgG and IgA antibodies against non-gluten proteins adds nothing to the pathologic relevance of these antibodies. Further research is needed on CXCR3-binding gliadin peptide antibodies as a possible biomarker and as a guide for dietary elimination in CD, Crohn's disease and a subgroup of children with ASD.

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1. Introduction

Immunologic reactivity to gluten proteins has been researched extensively in celiac disease and, to a much lesser degree, in Crohn's disease (Balakireva and Zamyatnin, 2016; Cimaglia et al., 2014; Schedel et al., 2005; Yang et al., 2005). Wheat in general comprises about 100 different proteins, the majority of which are alcohol-soluble, with the remainder being water-soluble (Chattopadhyay and Kumar, 2016; Kasarda et al., 2013; Kieffer et al., 1982; Ostergaard et al., 2000). Together, α-gliadins, γ-gliadins, ω-gliadins, and low and high molecular weight glutenins are the major alcohol-soluble proteins called gluten proteins, which represent about 75% of the total proteins of wheat grains (Yang et al., 2005). The remainder of wheat proteins, which are generally soluble in water or salt solutions, including serine protease inhibitors (serpins), purinins, farinins, α-amylase/protease inhibitors and globulins are called non-gluten proteins (Kenrick and Walker Smith, 1970; Moneret-Vautrin et al., 2011; Sotkovsky et al., 2008; Stern et al., 1979). Intestinal T cells from celiac disease (CD) patients respond to a heterogenous array of peptides derived from α-, γ-, ω-gliadins and glutenins, and produce a significant amount of interferon-γ (Camarca et al., 2009).

Interestingly, Camarca et al. showed that the immune system of some patients recognized many peptides from single or multiple gliadin families, while others reacted to only one peptide. This means that a large number of gluten epitopes may be involved in the development of gluten sensitivity, CD, and associated diseases. It is important to understand the nature and properties of immunodominant epitopes; not only can they aid in diagnosis, but they also have tolerance-related therapeutic applications in several T-cell-mediated diseases. The great majority of CD patients in the study (Camarca et al., 2009) reacted to at least one γ-gliadin-derived peptide, with half recognizing DQ2-γ-I. This suggests that the contribution of γ-gliadin peptides to CD pathogenesis may be greater than previously thought. Strong and frequent recognition was also found from the ω-gliadin-derived peptides (Arentz-Hansen et al., 2002; Camarca et al., 2009; Shan et al., 2002).

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Overall, 86% of the CD patients recognized a different array of peptides. This indicates that other gliadin peptides not tested in the study could be relevant in some CD patients (Camarca et al., 2009).

For this reason, immune response to non-gluten proteins of wheat was also investigated in celiac disease in a different study (Huebener et al., 2015). The results demonstrated that in addition to the well-recognized immune reaction to gluten proteins, celiac disease was also associated with humoral immune response directed against serpins, purinins, farinins, α-amylase/protease inhibitor and globulins. However, we found that blood samples from CD patients that reacted to gluten proteins uniformly also reacted to non-gluten proteins (Huebener et al., 2015). Apart from their involvement in CD, antibodies to these gluten and non-protein proteins have not been examined in the context of Crohn's disease and autism.

Crohn's disease and ulcerative colitis fall under the classification of inflammatory bowel disease (IBD). They are triggered by environmental factors, including microbial antigens and food (Huebener et al., 2015). The serologic response in Crohn's disease includes antibodies against specific components of *Saccharomyces cerevisiae*, mycobacteria, *Bacteroides*, and *Escherichia coli* (Barta et al., 2003; Giaffer et al., 1992; Knoflach et al., 1987; Main et al., 1988). In fact, the measurement of antibodies to baker's and brewer's yeasts directed against cell wall oligomannoside epitope (ASCA) has been proposed as a serological marker for Crohn's disease (Stern et al., 1979). These antibodies have a sensitivity of 60–70% for differentiating Crohn's disease from controls and a specificity of 80–95% (Quinton et al., 1988; Yang et al., 2005). Due to overlapping symptomatologies between CD and Crohn's disease, ASCA antibodies were also measured in a group of patients with CD. In patients with gluten sensitivity enteropathy (GSE), high incidences of ASCA were reported. This high prevalence of ASCA in CD patients stimulated us into investigating whether indeed these gluten and non-gluten proteins of wheat, in addition to playing a role in CD, also had some sort of involvement in Crohn's disease and autism.

Autism spectrum disorder (ASD) is a group of neuroimmune disorders in which genes and environmental triggers such as infections, toxic chemicals, and dietary components play a role. In our earlier study we measured antibodies against gliadin in children with ASD (Vojdani et al., 2003). Analysis of the blood samples revealed that a significant number of autistic children produced IgG and IgA antibodies against α-gliadin 33-mer peptide (Vojdani et al., 2003). Moreover, in a different study, the effectiveness of a gluten-free diet was tested on children with ASD, and a significant improvement in behavioral symptomatologies was observed in a subgroup (Elder et al., 2006). Similar to what was done with CD, this other study with ASD was conducted with α-gliadin 33-mer, but not with non-gluten or other gluten peptides, especially CXCR3-binding gliadin peptide.

CXCR3 is a chemokine receptor that is expressed in monocytes, eosinophils, NK cells, B cells, and T cells, particularly in $CD4^+$ TH1 cells (Groom and Luster, 2011). During the inflammatory process, CXCR3 promotes the recruitment of immune cells into the inflamed tissues by interacting with its three different ligands: CXCL9, CXCL10, and CXCL11. This CXCR3 interaction with its ligands becomes over-activated in different chronic inflammatory processes such as inflammatory bowel diseases, rheumatoid arthritis (Hosomi et al., 2011; Laragione et al., 2011; Lee et al., 2009), and in the small intestinal mucosa of untreated patients with CD (Bondar et al., 2014). The number of TH1 cells is increased in the duodenal mucosa of these patients, and these cells express an increased amount of CXCR3. Therefore, in the study just cited (Bondar et al., 2014), blood levels of soluble CXCL10 and CXCR3+ cells in duodenal biopsies were measured and found to be significantly elevated. Interestingly, CD patients on gluten-free diets presented levels of CXCL10 and numbers of CXCR3+ cells that were similar to those found in controls (Bondar et al., 2014). This over-activation of CXCR3 and its ligands and the infiltration of TH1 cells and plasma cells into the small intestine have been shown to be associated with two gliadin peptides that bind specifically to CXCR3 on the surface of these lymphocytes (Lammers et al., 2008).

Consequently, in this present study we tested IgG and IgA antibodies against the major gluten proteins, α-gliadin 33-mer, γ-gliadin 15-mer, glutenin 21-mer, and gliadin peptides that bind to CXCR3; and for the non-gluten wheat proteins, purinin, farinin, α-amylase, serpin and globulin. We examined IgG and IgA antibodies against these proteins in patients with CD, Crohn's disease and in children with ASD in comparison to healthy controls in order to find out whether or not measurements of humoral immune response against CXCR3-binding gliadin peptide and non-gluten proteins will add to the clinical efficacy of wheat proteome antibody testing in these disorders.

2. Experimental section

2.1. Materials and methods

2.1.1. Blood samples

Forty-eight sera from healthy control subjects aged 18–65 were obtained from Innovative Research (Novi, MI, USA). Commercially available sera of 24 patients with Crohn's disease and 24 sera from patients with CD were purchased from The Binding Site (San Diego, CA, USA), Inova (San Diego, CA, USA), Trina International Nanikon (Switzerland), Diamedix (Fl, USA) and Innovative Research (Novi, MI, USA). We also used sera from 48 children with ASD aged 2–15 that we had used in our earlier study that was approved by the Institutional Review Board of the Center for Autism and Related Disorders (Tarzana, CA, USA) and which had been stored at −80 °C.

The Crohn's disease sera samples were confirmed using the *Saccharomyces cerevisiae* (ASCA) IgA kit from Inova Diagnostics (San Diego, USA), and the degree of positive samples for celiac disease was determined by using gliadin IgA and transglutaminase-2 IgA kits also purchased from Inova Diagnostics (San Diego, USA).

The samples that were obtained from commercial sources were from regulated and certified providers who strictly maintain the anonymity of their sample donors and who are compliant with all required appropriate ethical practices.

The healthy subjects were tested according to FDA guidelines for the detection of hepatitis B surface antigen, antibodies to HIV, HIV-I RNA, Hepatitis-C RNA, and syphilis. All samples yielded non-reactive or negative results for each test performed.

2.1.2. Proteins and peptides

A whole-wheat antigen was prepared by combining water-soluble and alcohol-soluble proteins.

Different gliadin peptides including α-gliadin 33-mer, deamidated α-gliadin 33-mer, γ-gliadin 15-mer, glutenin 21-mer, CXCR3-binding gliadin peptides, purinin, farinin, serpin, and globulin peptides were synthesized by Bio-Synthesis Inc. (Lewisville, TX, USA). α-amylase inhibitor was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.3. Measurement of IgG and IgA by ELISA

Antigens and peptides from gluten proteins were dissolved in methanol, and the non-gluten proteins and peptides were dissolved in 0.1 M phosphate buffer saline (PBS) pH 7.4 at a concentration of 1.0 mg/mL, then diluted 1:100 in 0.1 M carbonate-bicarbonate buffer, pH 9.5. 100 μL each of the wheat mixture of water- and alcohol-soluble components were added to different

rows of a microtiter plate. Several wells were also coated with 2% of bovine serum albumin (BSA) or human serum albumin (HSA) and used as controls. Plates were incubated overnight at 4 °C and then washed three times with 200 μL Tris-buffered Saline (TBS) 0.05% Tween 20, pH 7.4. The non-specific binding of immunoglobulins was prevented by adding 200 μL of 2% BSA in TBS, and incubated overnight at 4 °C. Plates were washed as described above, and then serum samples diluted 1:50 for determination of IgA antibody and 1:100 for determination of IgG antibody in 1% BSA in TBS containing 0.05% Tween 20 were added to duplicate wells and incubated for 1 h at room temperature.

Plates were washed, and then alkaline phosphatase goat anti-human IgG or IgA F(ab')2 fragments (KPI, Gaithersburg, MD, USA) at an optimal dilution of 1:400 for IgA and 1:800 for IgG in 1% BSA-TBS were added to each appropriate well; plates were incubated for an additional 1 h at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100 μL of paranitrophenylphosphate in 0.1 mL diethanolamine buffer 1 mg/mL containing 1 mM MgCl2 and sodium azide pH 9.8. The reaction was stopped 45 min later with 50 μL of 1 N NaOH. The optical density (OD) was read at 405 nm with a microtiter plate reader. To exclude non-specific binding, the ODs of the control wells containing HSA or BSA were subtracted from all other wells. Sera from patients with celiac disease with known high titers of IgG and IgA against gliadin and transglutaminase-2 were used as positive controls. Additionally, the calibrators, negative and positive controls from Gliadin IgG and IgA ELISA kits from Inova Diagnostics (San Diego, USA) were used for additional levels of quality control and for examining the reproducibility of the ELISA assay.

2.1.4. Statistical methods used in the data analysis

The TTEST function in Microsoft Excel was used to determine the *P* values, comparing the data for the patients with the data for controls. These *P* values were then used to determine levels of significance.

3. Results

3.1. Number of patients and tests

The data for IgG and IgA antibodies against an array of gluten and non-gluten wheat antigens and peptides were derived from the sera of 48 healthy control subjects ages 18–65, 50% male and 50% female, with no history of GI disorder, including gluten sensitivity and inflammatory bowel disease. For comparison, these antibodies were also measured in 48 sera, which, based on elevations in gliadin and transglutaminase IgA (24 sera) and anti-*Saccharomyces* IgA (24 sera), were classified with the possibility of celiac disease and Crohn's disease, respectively. Also, IgG and IgA antibodies were tested in 48 children ages 2–15 who, based on the Diagnostic and Statistical Manual of Mental Disorders 5, were classified as having ASD.

3.2. Prevalence of IgG and IgA antibodies against gluten and non-gluten proteins in sera of healthy control subjects

We selected water-soluble and alcohol-soluble components of wheat to represent major antigens of wheat: four panels of peptides representing gluten proteins, and four peptides and one protein representing non-gluten components of wheat. In healthy controls we found that at 3 standard deviations (3SD) above the mean or 0.5 OD, 3 (Samples #2, 9 and 14) out of the supposedly healthy 48 control specimens reacted to IgG with reactions ranging from moderate to strong against the wheat mixture, gluten and non-gluten proteins. The strongest reaction was observed against the mixture of wheat proteins, followed by CXCR3-binding gliadin peptide, and then serpin or globulin. For simplification and clarity Fig. 1 shows results only for 24 out of the 48 control samples. Sample #2 reacted strongly against the wheat mixture, CXCR3-binding gliadin peptide and serpin, Sample #9 reacted strongly against the wheat mixture, α-gliadin 33-mer, CXCR3-binding gliadin peptide, purinin and serpin, and Sample #14 against the wheat mixture, α-gliadin 33-mer, and globulin. Sample #18 reacted weakly only against the wheat mixture, and Sample #24 reacted moderately to the wheat mixture and weakly against α-gliadin 33-mer and α-amylase. In relation to IgA immune reaction, Sample #9 reacted most against the wheat mixture, α-gliadin 33-mer, and CXCR3-binding gliadin peptide, to a lesser degree against serpin and globulin, and then weakest against glutenin, γ-gliadin, purinin, farinin and α-amylase inhibitor. Sample #14 reacted strongly against the wheat mixture and weakly against glutenin, CXCR3-binding gliadin peptide, and serpin. Sample #5 reacted weakly against the wheat mixture, α-gliadin 33-mer, glutenin CXCR3-binding gliadin peptide and serpin. Interestingly, out of 24 samples, Sample #7 reacted only against serpin (OD 0.76), Sample #11 reacted only against CXCR3-binding gliadin peptide (OD 0.98), #19 reacted only against α-amylase inhibitor (OD 0.55), and #22 reacted only against the wheat mixture (OD 0.61), as shown in Fig. 1 (IgA).

3.3. Detection of IgG and IgA antibodies against gluten and non-gluten proteins in the sera of children with ASD

IgG and IgA antibodies were measured against gluten and non-gluten proteins in 48 sera samples from children with ASD. Analysis of data showed that 16 out of 48 samples or about 33% of the samples reacted strongly with IgG against the mixture of wheat proteins and α-gliadin 33-mer. 12 or about 25% of the samples reacted with IgG against non-gluten proteins, and Sample #5 reacted weakly only with glutenin and serpin as shown in Fig. 2 (IgG). In this figure, only 24 out of 48 sample results are shown individually. In relation to IgA, 11 out of 48 samples or 23% showed a robust reaction against both gluten and non-gluten proteins with IgA immune reactivity against the mixture of wheat proteins being the strongest, followed by CXCR3-binding gliadin and serpin as shown in Fig. 2 (IgA).

3.4. Detection of IgG and IgA antibodies against gluten and non-gluten proteins in the sera of patients with Crohn's disease

IgG and IgA antibodies were measured against gluten and non-gluten proteins in 24 sera samples from patients with Crohn's disease. For IgG antibody, at the OD cutoff of 0.5, 11 out of 24 (46%) of the specimens reacted with the mixture of wheat proteins, and 9 specimens out of 24 (38%) reacted very strongly with both gluten and non-gluten proteins as shown in Fig. 3 (IgG). In comparison to IgG, the prevalence of IgA-positive specimens in patients with Crohn's disease was much lower. Over all, 6 out of 24 specimens (25%) reacted strongly with both gluten and non-gluten proteins, with the gluten protein CXCR3-binding gliadin peptide being the strongest.

3.5. Detection of IgG and IgA antibodies against gluten and non-gluten proteins in the sera of patients with celiac disease

IgG and IgA antibodies were measured against gluten and non-gluten proteins in 24 sera samples from patients with celiac disease. Results of these peptides and antigen recognition are illustrated in Fig. 4. At ELISA OD of 0.5 or 3SD above the mean, the value of IgG antibody was most reactive against CXCR3-binding gliadin peptides, followed by the mixture of wheat proteins and serpin.

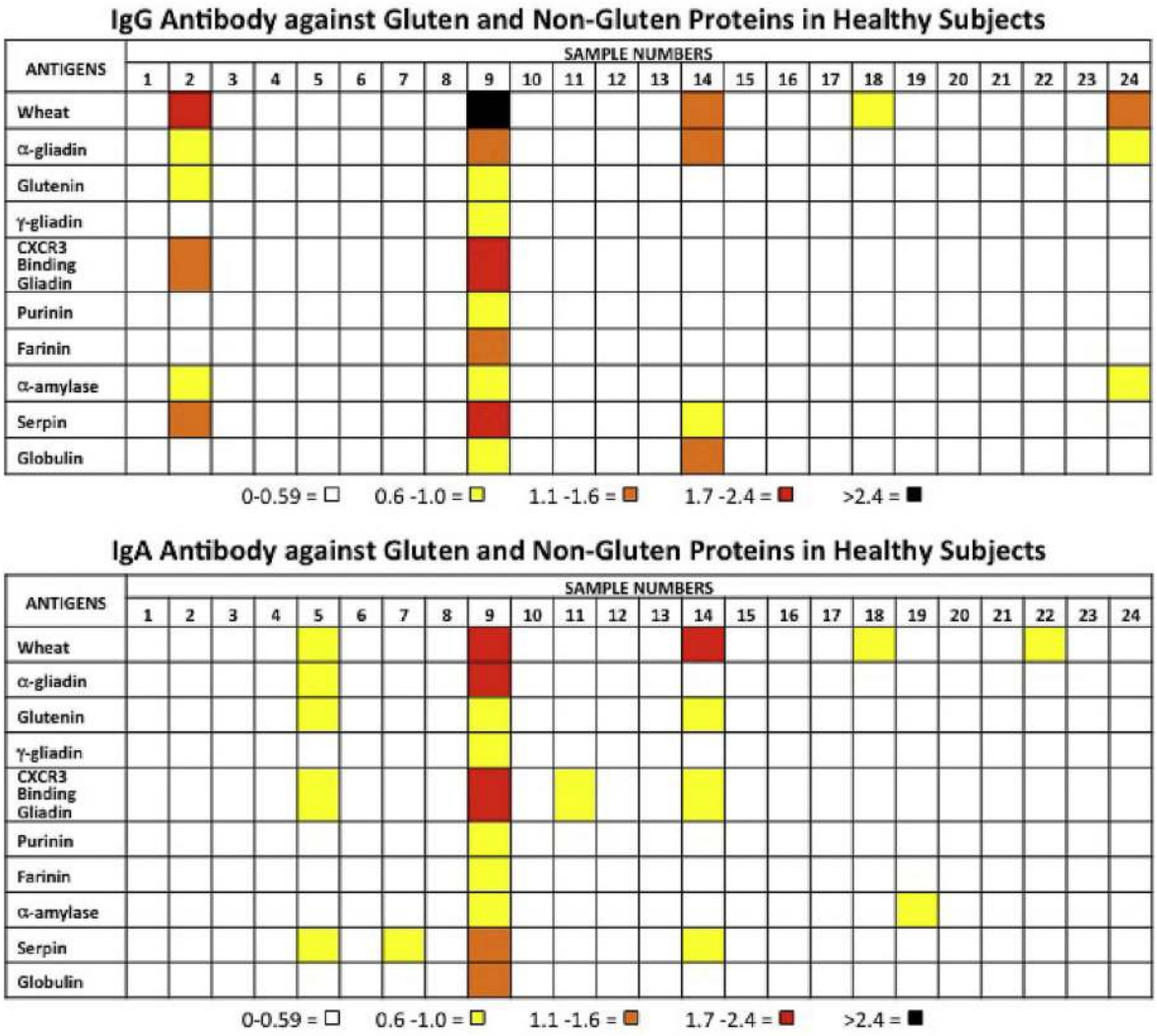

Fig. 1. Antibody against gluten and non-gluten proteins in healthy subjects. Only 24/48 individual test results are shown. (IgG) At 3SD above the mean of all healthy control subjects or 0.5 OD, 3 out of 24 specimens (Samples #2, 9 and 14) reacted with IgG from moderate to strong reaction against wheat, gluten and non-gluten proteins. The strongest reaction was observed against the mixture of wheat proteins, followed by CXCR3-binding gliadin, and then serpin or globulin. **(IgA)** Samples #9 and 14 reacted most against the mixture of wheat proteins, to a lesser degree against CXCR3-binding gliadin, and then weakest against the non-gluten protein, serpin. Similarly, Sample #5 reacted moderately against the wheat mixture and α-gliadin, followed by serpin and CXCR3-binding gliadin. Interestingly, out of 24 samples, only one (Sample #11) reacted only against CXCR3-binding gliadin, Sample #22 reacted only against the mixture of wheat proteins (OD 0.61), and Sample #19 had a very weak reaction against α-amylase (OD 0.55).

Overall, the majority of samples reacted strongly against both gluten or non-gluten proteins (Fig. 4, Table 1). The pattern of IgA antibodies against these same antigens and peptides was different from the pattern for IgG. All 24 specimens showed reactivity to more than one antigen or peptide. The most prominent reactions in descending order were against CXCR3-binding gliadin, α-gliadin 33-mer, serpin, purinin and then a mixture of wheat proteins as shown in Fig. 4 (IgA).

The statistical differences between the levels of IgG antibodies against a mixture of wheat proteins, gluten and non-gluten proteins comparing controls versus ASD, Crohn's and celiac disease groups are shown as means and *p* values in Table 1. The mean OD of IgG in control specimens for all 10 antigens varied from 0.22 ± 0.13 for γ-gliadin 15-mer to 0.48 ± 0.58 for the wheat protein mixture. In children with ASD the mean OD values for IgG were the lowest (0.26 ± 0.23) for γ-gliadin 15-mer as well, and 0.71 ± 0.82 for wheat mixture IgG, 0.43 ± 0.44 for α-gliadin 33-mer, and 0.53 ± 0.65 for CXCR3-binding gliadin peptide IgG. The *P* values for IgG antibody elevation against these 3 antigens were non-significant. Analysis of data from Crohn's disease patients showed the mean values for IgG against wheat mixture, α-gliadin 33-mer, purinin, farinin, α-amylase inhibitor, serpin, and globulin were the most significant ($P < 0.001$). Comparison of IgG data in controls versus CD were very significant for all tested 10 antigens ($P < 0.0001$). Moreover, the statistical differences between the mean of IgA antibodies in healthy controls versus children with autism were the most significant against α-gliadin 33-mer, γ-gliadin 15-mer, CXCR3-binding gliadin peptide, farinin, and α-amylase inhibitor ($P < 0.05$), but not with the other tested antigens. In comparison to controls, the IgA antibody against CXCR3-binding gliadin peptides was the most significant ($P < 0.0001$), followed by the wheat protein mixture, serpin and α-amylase inhibitor ($P < 0.001$) in patients with Crohn's disease. Finally, comparison of levels of IgA antibodies against a mixture of wheat proteins, gluten and non-gluten proteins comparing controls versus ASD, Crohn's and celiac disease groups shown as means and *p* values revealed robust immune reaction against all tested 10 antigens ($P < 0.0001$) with CXCR3-binding gliadin peptide, α-gliadin 33-mer, purinin and wheat protein

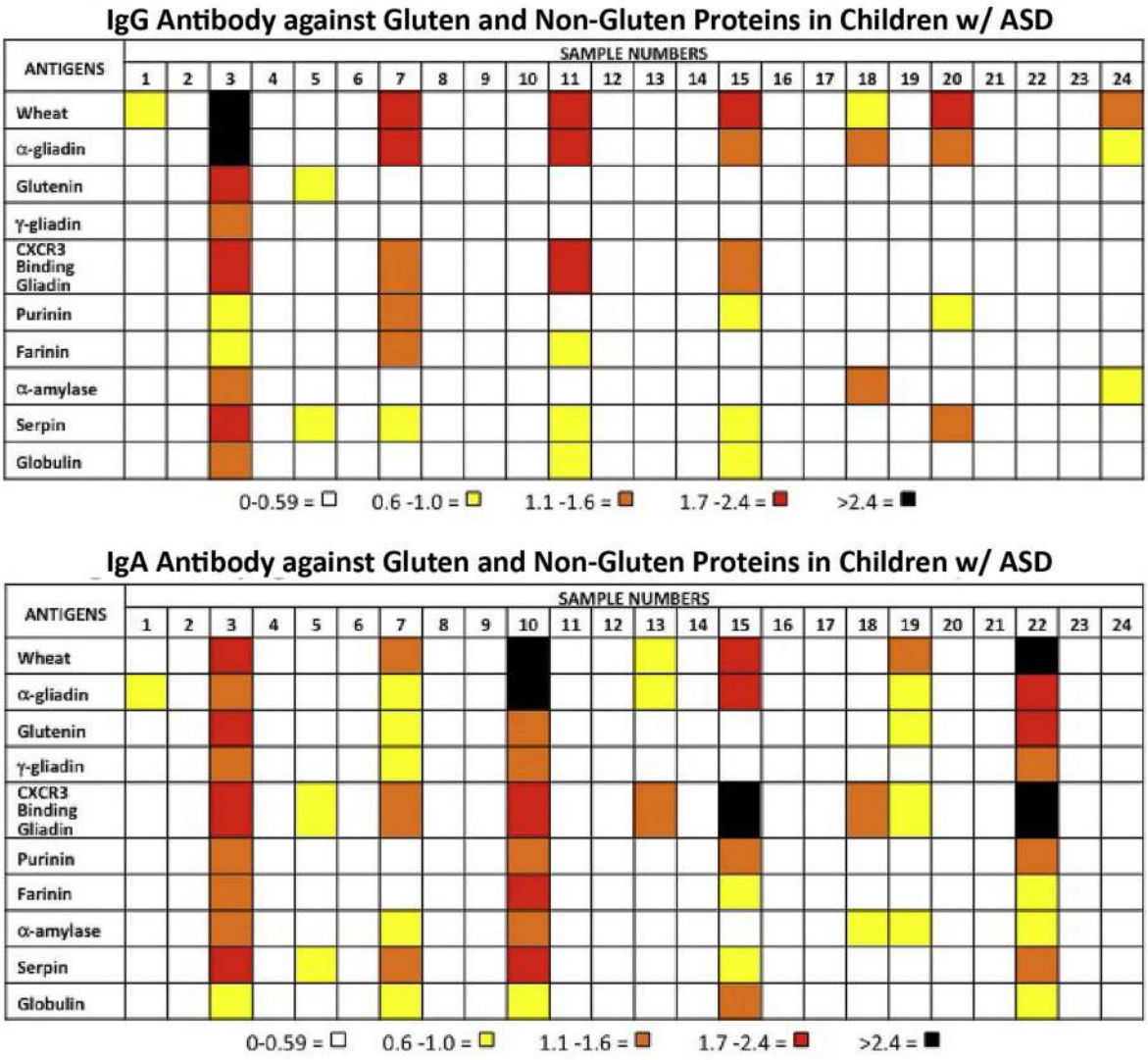

Fig. 2. Antibody against gluten and non-gluten proteins in children with autism spectrum disorder. Only 24/48 individual test results are shown. (IgG) 16 out of 48 samples or about 33% of the samples reacted strongly with IgG against the mixture of wheat proteins and α-gliadin. 12 or about 25% of the samples reacted with IgG against non-gluten proteins, and 1 sample (#5) reacted only with glutenin and serpin. **(IgA)** 11 out of 48 samples or 23% showed a robust reaction against both gluten and non-gluten proteins with IgA immune reactivity against a mixture of wheat proteins being the strongest, followed by CXCR3-binding gliadin and serpin.

mixture being the most significant (Table 2).

4. Discussion

The aim of this study was to examine the level and specificity of antibody response to gluten and non-gluten proteins, particularly CXCR3-binding gliadin peptide, in controls, children with ASD, patients with Crohn's disease and CD. By measuring IgG and IgA antibodies against gluten as well as non-gluten proteins in 144 blood samples from the above four groups, we asked the question whether or not testing antibodies against more antigens is better. Will it increase the specificity and pathogenic relevance of immunologic reactivity above the current testing methodologies for CD which use mainly α-gliadin 33-mer peptide? To answer this we selected the major proteins of wheat: α-gliadin 33-mer, γ-gliadin 15-mer, glutenin 21-mer and CXCR3-binding gliadin peptide to represent gluten proteins; and purinin, farinin, α-amylase inhibitor, serpin and globulin as the major non-gluten proteins. It has been shown in earlier studies that intestinal T cells from patients suffering from gluten sensitivity or celiac disease responded to a heterogenous array of peptides from α-gliadin, γ-gliadin, ω-gliadin and glutenins (Arentz-Hansen et al., 2002; Camarca et al., 2009; Shan et al., 2002).

A recent study demonstrated that in addition to the well-recognized immune reaction to gluten peptides, CD could be associated with strong humoral response directed at specific non-gluten proteins of wheat such as purinin, farinin, α-amylase inhibitor, serpin and globulin (Huebener et al., 2015). However, the study showed that a patient with celiac disease who reacted strongly to non-gluten proteins would also react to gluten proteins.

In an earlier study conducted in our lab (Vojdani et al., 2014) we tested IgG, IgM and IgA antibodies against wheat proteins, α-gliadin 33-mer and γ-gliadin 15-mer in samples from 400 different blood donors. We concluded that a subgroup of up to 16% of blood donors, due to breakdown of immunological tolerance, may react and produce significant levels of antibodies against wheat and gluten proteins (Hosomi et al., 2011). Although the number of tested samples from blood donors in this present study was not as extensive as in the earlier one, we found that 17% of the current study's healthy controls reacted strongly to both gluten and non-

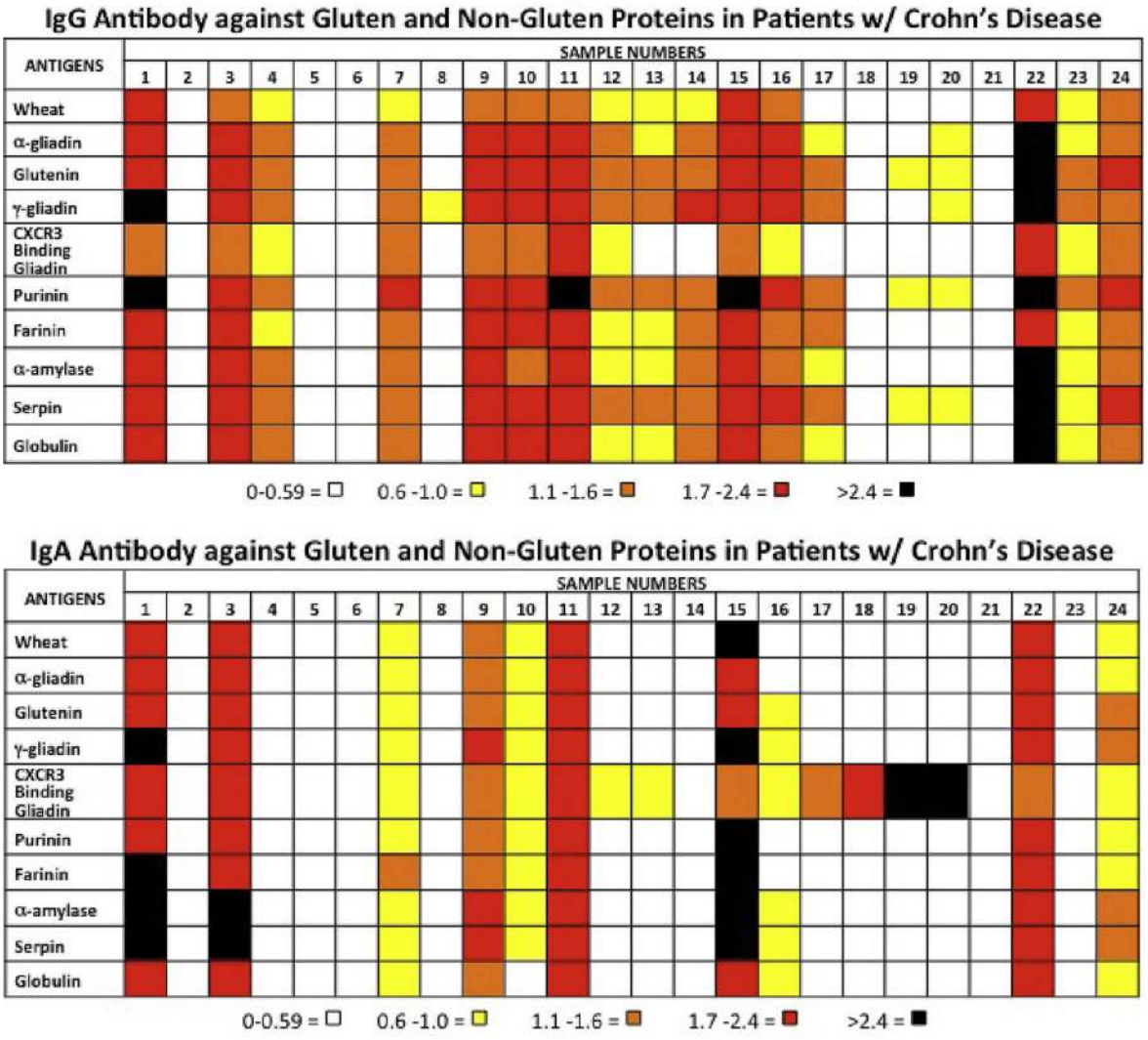

Fig. 3. Antibody against gluten and non-gluten proteins in patients with Crohn's disease. (IgG) 11 out of 24 (46%) of the specimens reacted with wheat, and 9 specimens out of 24 (38%) reacted very strongly with both gluten and non-gluten proteins. (**IgA)** 6 out of 24 specimens (25%) reacted strongly with both gluten and non-gluten proteins, with the gluten protein CXCR3-binding gliadin peptide being the strongest. 8 (33%) of the samples did not exhibit any IgA antibody against the wheat antigens, gluten and non-gluten peptides.

gluten proteins IgG, while 10% reacted strongly for IgA (Fig. 1). Testing for non-gluten proteins did not add any information to the antigenic specificity and the immunologic reactivity in the controls. We then analyzed the data from the sera of children with ASD, and found that up to 33% of the blood samples reacted strongly against gluten and non-gluten proteins (Fig. 2). Again we observed that strong immune reaction against non-gluten proteins correlated with reaction against gluten proteins, except for Sample #13, which was the only one that in fact produced IgA antibody against gluten proteins, but not against non-gluten proteins (Fig. 2-IgA). We noted with interest that among the supposedly healthy control samples, Patient 9 showed very strong reactivity with both IgG and IgA antibodies against the wheat protein mixture, CXCR3-binding gliadin peptide, α-gliadin 33-mer, and, to a lesser extent, serpin and globulin (Fig. 1). We reiterate that this could indicate that Patient 9 may actually have what is variously known as silent, hidden, or the early stages of CD and NCGS.

Due to some symptomatology overlap between Crohn's disease and CD, we applied IgG and IgA measurements against various wheat antigens and associated peptides to the sera of patients with Crohn's disease to examine the possibility of immune reaction to non-gluten proteins but not to gluten proteins. In comparison with healthy controls, IgG antibody in the sera of patients with Crohn's disease was found to be highly elevated against antigens from both gluten and non-gluten proteins in 38% of tested specimens. Similarly, IgA antibody reactivity was strong against both gluten and non-gluten proteins (Fig. 3). Finally, Fig. 4 presents data from CD patients with antibodies against gluten proteins, which have been demonstrated to be closely associated with CD and are widely utilized as serologic markers of this condition (Schedel et al., 2005); in this group, IgG and, especially, IgA antibodies were detected strongly against both gluten and non-gluten proteins. The robust IgA response was greatest against CXCR3-binding gliadin peptide, followed by the α-gliadin 33-mer peptide, and then to a lesser degree against serpin and other gluten and non-gluten proteins (Fig. 4).

Huebener et al. showed in their study (Huebener et al., 2015) that compared with healthy controls, patients with CD exhibited higher levels of antibody reactivity to non-gluten proteins. Our own data from CD patients is in full agreement with their findings that

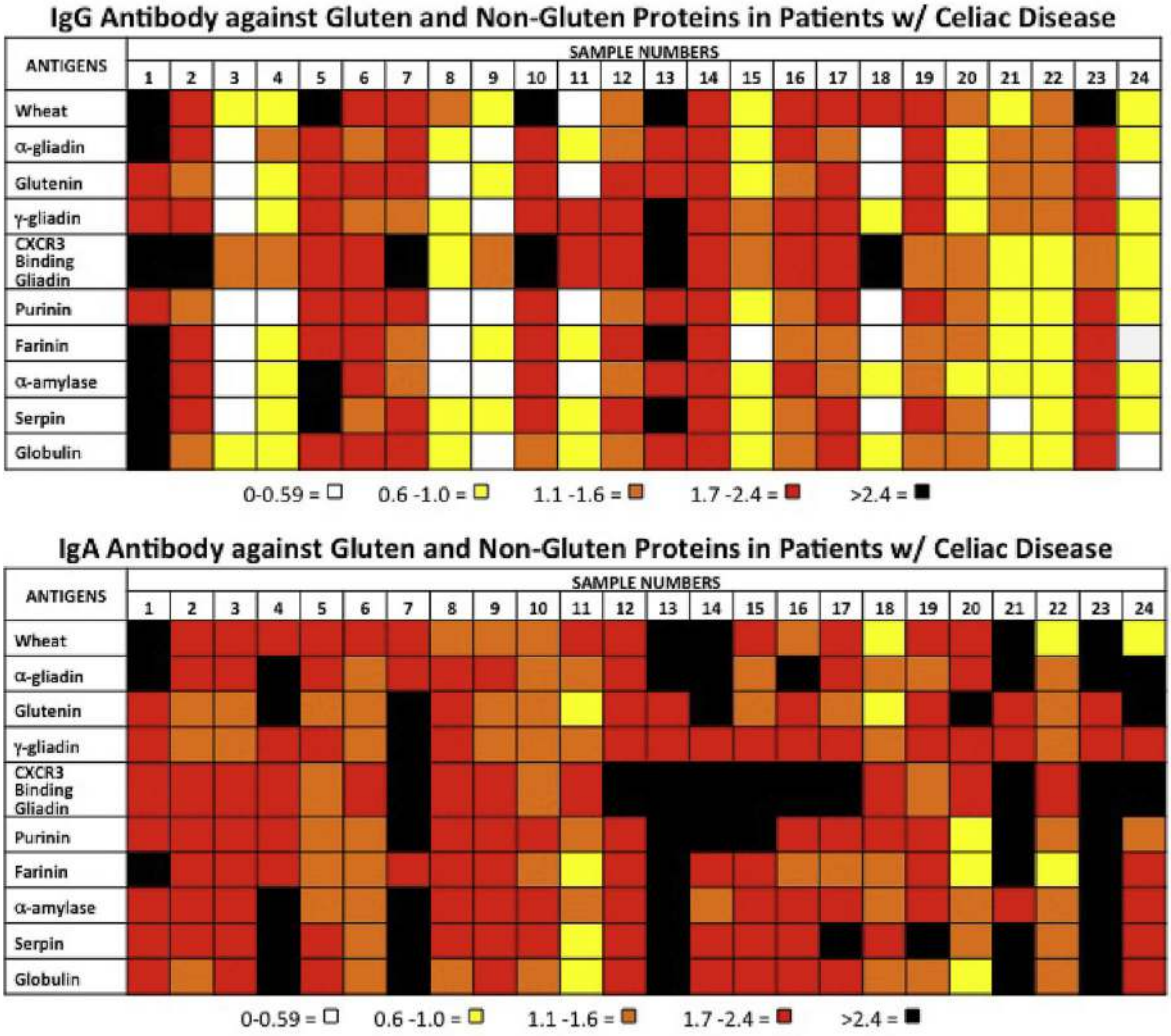

Fig. 4. Antibody against gluten and non-gluten proteins in patients with celiac disease. (IgG) At ELISA OD of 0.5 or 3SD above the mean, the value of IgG antibody was most reactive against CXCR3-binding gliadin peptides, followed by the mixture of wheat proteins and serpin. **(IgA)** All 24 specimens showed reactivity to more than one antigen or peptide. Overall, the majority of samples reacted strongly against both gluten or non-gluten proteins.

non-gluten proteins of wheat are indeed additional target antigens in celiac disease; however, based on our data from controls, children with ASD, and patients with CD and Crohn's disease, we did not observe that non-gluten proteins are novel target antigens (Barta et al., 2003). This is because in all 144 tested blood specimens, as previously stated, we found that if a sample reacted against gluten proteins it would also react strongly against non-gluten proteins. This similarity in IgG and IgA response against gluten and non-gluten proteins may be related to the possible cross-reactivity and epitope similarities between the two groups of proteins (Huebener et al., 2015).

For example, a homology analysis indicates that γ-gliadins are close in sequence to purinin proteins. Short sequences of α-amylase/protease inhibitors and the farinin group of proteins have been found to be similar to those in certain γ-gliadin and low molecular weight glutenin proteins. In addition, the reactive centers of some of the identified serpin antigens share homology with glutamine-rich repeats in gluten proteins such as CXCR3-binding gliadin peptide (Lammers et al., 2008; Ostergaard et al., 2000).

Indeed, we observed a robust IgA immune response against CXCR3-binding gliadin peptide, α-gliadin 33-mer, then the mixture of wheat proteins, followed by the other gluten and non-gluten proteins. This robust immune response against CXCR3-binding gliadin peptide merits great interest, since it has been shown that due to its resistance to digestion and its binding to CXCR3, it activates the inflammatory cascade that induces an increase in both intestinal permeability and zonulin release, which is the hallmark of CD (Lammers et al., 2008).

Therefore, additional research should be conducted on a much larger number of specimens from various GI disorders in order to examine the possibility of adding the anti-CXCR3-binding gliadin peptide to the current repertoire of gluten antibody testing, not only in patients with CD, but with ASD and Crohn's disease and possibly other disorders.

5. Conclusions

The results of this study demonstrate that although humoral immune response to wheat proteins in children with ASD and patients with Crohn's disease and CD is not limited only to gluten proteins, measuring IgG and IgA antibodies against non-gluten proteins does not add anything to the pathologic relevance of these antibodies. In fact, measuring IgG and IgA antibodies against gluten proteins, in particular, CXCR3-binding gliadin peptide is

Table 1
The statistical differences between the levels of IgG antibodies against a mixture of wheat proteins, gluten and non-gluten proteins comparing controls versus ASD, Crohn's and CD groups are shown as means and *p* values. The patterns of reactivity to the gluten and non-gluten proteins differ from one disorder to another. In the CD and ASD groups the strongest reactions were with CXCR3-binding gliadin, followed by the wheat protein mixture. In the Crohn's group the strongest reaction was to the wheat mixture.

	Control	ASD	Crohn's	Celiac
Wheat				
Mean	0.48	0.71	0.76	1.20
p values		0.1404	0.0010	0.0001
α-Gliadin				
Mean	0.42	0.43	0.65	0.84
p values		0.4554	0.0010	0.0001
Glutenin				
Mean	0.25	0.32	0.45	0.77
p values		0.1837	0.0500	0.0001
γ-Gliadin				
Mean	0.22	0.26	0.46	0.94
p values		0.2424	0.0200	0.0001
CXCR3 b gli				
Mean	0.29	0.53	0.43	1.23
p values		0.0813	0.0500	0.0001
Purinin				
Mean	0.27	0.35	0.73	0.71
p values		0.1441	0.0010	0.0001
Farinin				
Mean	0.26	0.31	0.61	0.86
p values		0.2594	0.0010	0.0001
α-Amylase inhibitor				
Mean	0.27	0.35	0.65	0.91
p values		0.1832	0.0010	0.0001
Serpin				
Mean	0.36	0.43	0.75	1.10
p values		0.3009	0.0010	0.0001
Globulin				
Mean	0.26	0.29	0.59	0.82
p values		0.3253	0.0010	0.0001

Table 2
The statistical differences between the levels of IgA antibodies against a mixture of wheat proteins, gluten and non-gluten proteins comparing controls versus ASD, Crohn's and celiac disease groups are shown as means and *p* values. All three disease groups reacted strongest against CXCR3-binding gliadin.

	Control	ASD	Crohn's	Celiac
Wheat				
Mean	0.38	0.74	0.48	1.27
p values		0.0557	0.0010	0.0001
α-Gliadin				
Mean	0.31	0.63	0.46	1.42
p values		0.0344	0.0500	0.0001
Glutenin				
Mean	0.25	0.45	0.41	1.25
p values		0.0678	0.0500	0.0001
γ-Gliadin				
Mean	0.18	0.42	0.50	1.10
p values		0.0132	0.0010	0.0001
CXCR3 b gli				
Mean	0.33	0.77	0.79	1.43
p values		0.0200	0.0001	0.0001
Purinin				
Mean	0.22	0.37	0.44	1.31
p values		0.0569	0.0500	0.0001
Farinin				
Mean	0.17	0.34	0.48	1.15
p values		0.0377	0.0500	0.0001
α-Amylase inhibitor				
Mean	0.24	0.40	0.57	1.12
p values		0.0359	0.0010	0.0001
Serpin				
Mean	0.28	0.48	0.59	1.26
p values		0.0872	0.0010	0.0001
Globulin				
Mean	0.23	0.34	0.39	1.10
p values		0.1142	0.0600	0.0001

sufficient to detect celiac disease with high specificity and sensitivity. Therefore, measuring more isn't always better.

Conflicts of interest

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