

## Anti-microbial antibodies in celiac disease: Trick or treat?

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### Abstract

**AIM:** To determine the prevalence of a new set of anti-glycan and anti-outer membrane protein (anti-OMP) antibodies in a Hungarian cohort of adult Celiac disease (CD) patients.

**METHODS:** 190 consecutive CD patients [M/F: 71/119, age:39.9 (SD:14.1) years], 100 healthy, and 48 gastrointestinal controls were tested for glycan anti-

Saccharomyces cerevisiae (gASCA), anti-laminaribioside (ALCA), anti-chitobioside, anti-mannobioside, anti-OMP antibodies and major NOD2/CARD15 mutations. Thirty out of 82 CD patients enrolled at the time of diagnosis were re-evaluated for the same antibodies after longstanding gluten-free diet (GFD).

**RESULTS:** 65.9% of the CD patients were positive for at least one of the tested antibodies at the time of the diagnosis. Except anti-OMP and ALCA, anti-microbial antibodies were exclusively seen in untreated CD; however, the overall sensitivity was low. Any glycan positivity (LR+: 3.13; 95% CI: 2.08-4.73) was associated with an increased likelihood ratio for diagnosing CD. Significant correlation was found between the levels of anti-glycan and anti-endomysial or anti-transglutaminase antibodies. Anti-glycan positivity was lost after longstanding GFD. Anti-glycan antibody titers were associated with symptoms at presentation, but not the presence of NOD2/CARD15 mutations. Patients with severe malabsorption more frequently had multiple antibodies at diagnosis ( $P = 0.019$ ).

**CONCLUSION:** The presence of anti-glycan antibodies in CD seems to be secondary to the impaired small bowel mucosa which can lead to increased antigen presentation. Furthermore, anti-glycan positivity may be considered an additional marker of CD and dietary adherence.

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**Key words:** Celiac disease; Glycans; Anti-Saccharomyces cerevisiae antibodies; Anti-outer membrane protein antibody; NOD2/CARD15; Gluten-free diet; Presenting symptoms; Bacterial translocation; Crohn's disease

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## INTRODUCTION

Celiac disease (CD) is a genetically determined chronic inflammatory disorder with autoimmune components induced by exposure to gluten<sup>[1]</sup>. The clinical presentation of the disease is highly variable and little is known about the factors that determine the type of symptoms. The inflamed and damaged small bowel mucosa as well as the clinical symptoms shows recovery in most affected subjects after a complete removal of gluten from their diet<sup>[2]</sup>. The presence of different autoantibodies is a typical feature of CD; however, the exact mechanism behind their production and potential role in the disease pathogenesis has not yet been fully understood<sup>[3]</sup>. Tissue transglutaminase 2 (TG2) is the main autoantigen of anti-endomysial antibodies (EMA) and anti-TG2/EMA are commonly used for screening and diagnosing CD<sup>[4]</sup>. More recently antibodies directed against synthetic deamidated gliadin peptides have also been suggested as a reliable tool for diagnosing CD<sup>[5]</sup>. Most of the other antibodies do not appear to have either high sensitivity or specificity for CD, but they may be associated with specific clinical presentations or extra-intestinal manifestations<sup>[6]</sup>. As a sign of the cytoskeleton and intercellular tight junction involvement, a high prevalence of IgA anti-actin antibodies was also reported which strongly correlated with the degree of villous atrophy, appearing in more severe forms of the disease<sup>[7]</sup>. Furthermore, production of anti-actin antibodies was gluten dependent. After strict adherence to gluten-free diet (GFD), they become undetectable within several months indicating that their appearance follows mucosal injury<sup>[8,9]</sup>. Similarly, also the occurrence of anti-zonulin antibodies was associated with active CD and disappearing during remission<sup>[10]</sup>.

The presence of serological responses to various microbial antigens [e.g. phosphopeptidomannan cell-wall component of anti-Saccharomyces cerevisiae (ASCA), outer membrane porin C transport protein of the *Escherichia coli* (OmpC) or the *Pseudomonas fluorescens* associated protein I2] is characteristic of Crohn's disease and has been intensively studied for its clinical value in this patient group<sup>[11]</sup>. The occurrence and magnitude of the seroreactivity are associated with complicated small bowel disease and the need for surgical intervention<sup>[12,13]</sup>. More recently, antibody formations against different glycans, which are common structures in the glycocalyx of pathogenic yeast and bacteria<sup>[14]</sup>, have also been reported in this patient group. Our group demonstrated that with the use of anti-glycan (g)ASCA and a panel of novel anti-glycan antibodies [anti-mannobioside (AMCA), anti-laminaribioside (ALCA), and anti-chitobioside (ACCA)], gave the same conclusions. Additionally, we evaluated the performance of all four anti-glycan antibodies and the traditional combination of ASCA IgG and IgA. Both panels equally identified 59.4% of all Crohn's disease patients. Eighty percent of these identified cases were the same patients while the remaining 10%-10% were detected by only one of the panels<sup>[15]</sup>.

Despite the fact that the exact mechanism behind antibody formation or their possible relevance in the

pathogenesis still needs to be elucidated, current data suggest that these markers are not an epiphenomenon related to the inflamed, leaky bowel mucosa<sup>[16-18]</sup> but reflect a loss of tolerance toward bacterial and fungal flora<sup>[19]</sup>. Furthermore, the anti-microbial antibodies might represent genetic susceptibility because patients who have positive antibodies often carry mutations in the NOD2/CARD15 gene<sup>[11,20,21]</sup>.

Anti-microbial antibody formation has also been reported in CD. ASCAs remain the most widely investigated antibodies<sup>[22-26]</sup> in this patient group but increasing experimental data are available on newly discovered antibodies such as anti-I2 or anti-OmpW (*Bacteroides caccae* TonB-linked outer membrane protein)<sup>[27,28]</sup>. The frequency of ASCA varied from 27% to 59% in various studies, owing to the differences in the number and the age of the patients as well as the commercial assays used for antibody detection. The frequency of seropositivity and serum levels of these antibodies clearly decreased during GFD.

We hypothesized that newly discovered inflammatory bowel disease (IBD)-associated antibodies (including anti-glycan antibodies and anti-OMP) may also be of importance in CD and aimed to determine the prevalence of these antibodies in a Hungarian cohort of adult CD patients in relation to clinical presentation, GFD and NOD2/CARD15 mutations.

## MATERIALS AND METHODS

### Patients

One-hundred and ninety consecutive, unrelated Hungarian adult patients with biopsy-proven CD (male/female: 71/119, mean age: 39.9 years, SD: 14.1) and 66 of their first degree relatives (siblings, mean age: 37.7 years, SD: 13.9) were investigated. The diagnosis of CD was based on small bowel biopsy showing severe villous atrophy with crypt hyperplasia (Marsh type III lesions)<sup>[29]</sup> and elevated serum levels of antibodies against transglutaminase (TGA) and endomysium (EMA). IgA and IgG class EMA were investigated on human umbilical cord substrate using indirect immunofluorescence method as previously described<sup>[30]</sup>. TGA were measured by enzyme-linked immunosorbent assay (ELISA) using human recombinant antigen expressed in *Escherichia coli*<sup>[31,32]</sup>.

Of the 190 patients, 82 patients' sera were obtained at the time of diagnosis (Group CD1) and in 30 of these 82 patients further serum samples were re-evaluated for the same antibodies after adherence to long-standing GFD. The median follow-up period between these blood samplings was 28.5 mo [interquartile range (IQR): 18-52]. In the 108 remaining cases the diagnosis of CD had been established prior to this study and they adopted a strict GFD. These 108 patients were divided into two separate groups according to their current TGA and EMA status and dietary compliance at the time of the sampling. Thirty-three patients still had positive EMA and TGA results (Group CD2) and the median duration was here 3.5 mo (IQR: 1-11). The adequate compliance was indicated by reduced antibody titers as compared to those at diagnosis. The remaining 75 patients had

negative EMA and normal TGA titers (Group CD3), median follow up: 21 mo (IQR: 6-85).

Detailed clinical data concerning the clinical presentation of CD at diagnosis were classified as follows: (1) severe generalized malabsorption (presence of at least four of the following five symptoms: diarrhea, abdominal distension, weight loss, anemia, hypoproteinemia); (2) non-specific gastrointestinal symptoms that did not compromise the general condition (diarrhea, constipation, bloating, recurrent abdominal pain or vomiting, esophageal reflux); (3) iron deficiency anemia without major gastrointestinal complaints; (4) dermatitis herpetiformis; (5) silent disease (population screening); (6) other (autoimmune diseases, reduced bone mineral density, liver disease, brain disease). Patients were assigned to one of these major presentation types in a prospective manner, based on clinical and routine laboratory results at diagnosis.

The control group consisted of 100 healthy, ethnically similar, blood donor individuals (male/female: 47/53, mean age: 36.6 years, SD: 9.1) who had normal findings on a thorough medical examination, blood pressure measurements, and routine laboratory tests. A second non-celiac gastrointestinal disease control group consisted of 48 patients with irritable bowel syndrome/diverticulosis without inflammation (male/female: 21/26, mean age 40.4 years, SD: 16.1). In controls, CD was excluded by the negativity of EMA and TGA. None of the control subjects had a family history of CD. Further comparisons were made with the previously published Crohn's disease cohort we investigated for the same antibodies earlier<sup>15</sup>.

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the University of Debrecen (DEOEC RKEB/IKEB: 2600-2007). Informed consent was obtained from all patients and controls.

#### **Anti-microbial antibody assays**

gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA (IBDX<sup>®</sup>, Glycominds Ltd., Lod, Israel), ASCA IgG, ASCA IgA and OMP IgA (QUANTA Lite<sup>™</sup> OMP PLUS, INOVA Diagnostics, San Diego, CA) were measured in sera according to the manufacturers' protocols. The results were presented as arbitrary units, which were calculated based on sample and calibrator optical density. Cut-off levels used for the determination of positivity were according to the manufacturers' guidelines: 50, 100, 60 and 90 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA, respectively, and 25 U/mL for ASCA IgG, ASCA IgA and OMP IgA.

#### **Detection of NOD2/CARD15 mutations**

One-hundred and thirty-four CD patients and 100 healthy control subjects were eligible for NOD2/CARD15 mutation analysis. Major NOD2/CARD15 mutations (SNP8, 12 and 13) were determined as previously described<sup>11,31</sup> by denaturing high-performance liquid chromatography (dHPLC, Wave DNA Fragment Analysis System, Transgenomic Limited, UK). Sequence variation, observed in the dHPLC profile, was sequenced on both

strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA) and samples were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

#### **Statistical analysis**

Variables were tested for normality with Shapiro Wilk's  $W$  test.  $t$ -test with separate variance estimates,  $\chi^2$ -test,  $\chi^2$ -test with Yates correction and likelihood ratio (LR) test were calculated to evaluate differences between various groups of CD patients and controls, as well as within subgroups of CD patients, as appropriate. Sensitivities, specificities, positive and negative likelihood ratios were calculated to determine the predictive power of gASCA, AMCA, ALCA, ACCA, OMP or the combination of these markers in distinguishing between CD and controls. Spearman's rank order correlation was calculated to test the association between anti-glycan/OMP and TGA levels. Two-sided  $t$ -test for independent samples with separate variance estimates and ANOVA with post hoc Scheffe test were used to analyze the association between anti-glycan antibody titers and clinical symptoms at diagnosis. A  $P$  value of  $< 0.05$  was considered as significant. For statistical analysis, SPSS15.0 (SPSS Inc, Chicago, IL) was used with the help of a statistician (Dr. Peter Vargha).

## **RESULTS**

### **Presence of anti-glycan and anti-OMP antibodies at the time of diagnosis of celiac disease**

The frequency and the mean titers of gASCA IgG, AMCA IgG, and ACCA IgA were significantly higher at the time of diagnosis of CD than in healthy and non-celiac gastrointestinal control groups (Table 1, Figure 1). However, the frequency of ALCA IgG and anti-OMP IgA positivity and also the mean titers in the patients were similar to those in control groups. No difference was found between healthy subjects and GI controls based on the presence of these antibodies. For that reason, we only used the healthy subjects as a control group in the subsequent comparisons.

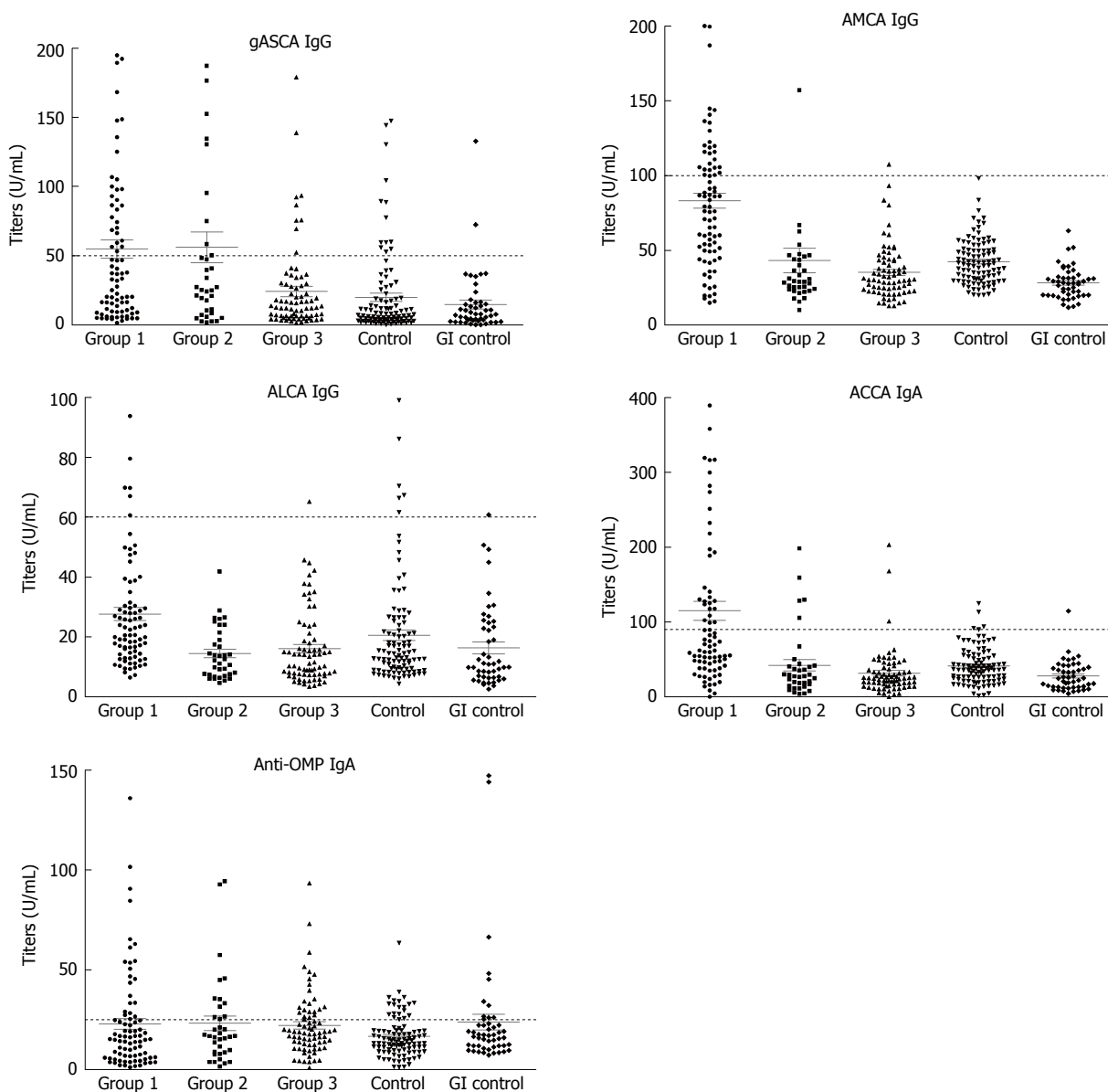
When calculating the sensitivity and specificity of the different markers based on the cut-off values suggested by the manufacturer, 65.9% of the CD patients were positive for at least one of the tested anti-microbial antibodies at the time of diagnosis. Except ALCA, all anti-glycan antibodies were specific for untreated CD. However, the overall sensitivity was low (gASCA: 39.0%, AMCA: 35.4%, ACCA: 37.8%). The above association was further tested by using the LR test. The sensitivity, specificity, positive and negative LR between CD at diagnosis and controls are presented in Table 2. Compared to healthy controls, gASCA, AMCA, and ACCA were associated with a moderate increase in the likelihood of CD, respectively. The positivity of any anti-glycan antibody significantly increased the likelihood for untreated CD (Table 2).

Detailed clinical data on the symptoms at the time

**Table 1** Frequency of anti-microbial antibodies in 190 patients with celiac disease and in control groups *n* (%)

	Group 1	Group 2	Group 3	Control	GI control
<i>n</i>	82	33	75	100	48
TGA IgA (U/mL) (median; IQR)	54.3 (20.2-100.0)	32.2 (9.9-73.1)	2.7 (2.2-3.7)	-	-
gASCA IgG positive	32 (39.0) <sup>b,f,j</sup>	12 (36.3) <sup>d,f,i</sup>	10 (13.3)	14 (14)	2 (4.2)
AMCA IgG positive	29 (35.4) <sup>b,f,j,n</sup>	3 (9.1)	1 (1.3)	0 (0)	0 (0)
ALCA IgG positive	7 (8.5)	0 (0)	0 (0)	6 (6)	1 (2.1)
ACCA IgA positive	31 (37.8) <sup>b,f,j,n</sup>	4 (12.1)	3 (4.2)	6 (6)	1 (2.1)
Any glycan positive	54 (65.9) <sup>b,f,j,p</sup>	15 (45.4) <sup>d,f,i</sup>	15 (20)	21 (21)	4 (8.4)
Anti-OMP IgA positive	22 (26.8)	11 (33.3)	23 (30.7)	20 (20)	11 (22.9)

TGA: Antibodies against transglutaminase; Group 1: Celiac patients at the time of diagnosis; Group 2: Celiac patients after starting a gluten-free diet but still with celiac antibody positivity; Group 3: Celiac patients on a long-term strict gluten-free diet; Control: Healthy control; GI control: Non-celiac gastrointestinal disease control. Cut-off levels used for the determination of positivity were according to the manufacturers' guidelines: 50, 100, 60, 90 U/mL and 25 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA, and anti-OMP IgA, respectively. <sup>b</sup>*P* < 0.001, <sup>d</sup>*P* < 0.01, group 1 or Group 2 vs control; <sup>f</sup>*P* < 0.001, group 1 or Group 2 vs GI control; <sup>i</sup>*P* < 0.001, <sup>j</sup>*P* < 0.01, group 1 or Group 2 vs Group 3; <sup>n</sup>*P* < 0.001, <sup>p</sup>*P* < 0.03, group 1 vs Group 2. Using  $\chi^2$ -test with Yates correction.



**Figure 1** Anti-microbial antibody levels in 190 patients with celiac disease and in control groups. Individual values are shown by black spots. Mean values with standard error bars are indicated in gray. Cut-off values for positivity are pointed out by dotted line and 50, 100, 60, 90 and 25 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA and OMP IgA, respectively.

**Table 2** Predictive power of serological markers for distinguishing between patients with celiac disease at the time of diagnosis and various control groups

	Sensitivity (%)	Specificity (%)	95% CI	
			LR+	LR-
Celiac disease <i>vs</i> healthy controls				
gASCA	39	86	2.73 (1.57-4.76)	0.71 (0.59-0.86)
AMCA	35	100	-	-
ACCA	38	94	6.36 (2.76-14.4)	0.66 (0.56-0.79)
Any glycans	66	79	3.13 (2.08-4.73)	0.43 (0.31-0.59)
Celiac disease <i>vs</i> non-celiac gastrointestinal controls				
gASCA	39	96	9.36 (2.35-37.4)	0.64 (0.53-0.76)
AMCA	35	100	-	-
ACCA	38	98	18.1 (2.56-128.5)	0.64 (0.53-0.76)
Any glycans	66	92	7.90 (3.05-20.4)	0.37 (0.27-0.51)

**Table 4** Occurrence of multiple antibody responses to microbial antigens in untreated celiac disease patients in relation to the number of responses against microbial antigens *n* (%) (*n* = 78)

	0	1	2 to 4	Total
Severe malabsorption	7 (22)	8 (25)	17 (53)	32 (40)
Non-specific gastrointestinal symptoms	13 (38)	12 (35)	9 (27)	34 (44)
Iron deficiency anemia	6 (67)	3 (33)	0	9 (12)
Others	2	0	1	3 (4)
Total	28 (36)	23 (29)	27 (35)	78 (100)

*P* = 0.019 by  $\chi^2$ -test. Clinical data were not available for 4 patients.

of diagnosis in Group CD1 was available in 78 patients out of 82. Of the 78 patients, 32 (41%) presented with severe malabsorption, 34 (43.6%) with non-specific or minor gastrointestinal symptoms, 9 (11.5%) with iron deficiency anemia, and 3 (3.9%) with other symptoms. The titers of the anti-glycan antibodies varied according to the presenting symptoms (Table 3) by 2-sided *t*-test for independent samples with separate variance estimates. If the above association was tested by ANOVA and post hoc Scheffe-test only the association for gASCA (*P* = 0.027) and AMCA (*P* = 0.03) remained significant. Moreover, the clinical presentations of CD were distributed differently according to serological response (Figure 2, Table 4). Patients with severe malabsorption more frequently had multiple antibodies (*P* = 0.019) while in those with non-specific gastrointestinal symptoms or iron deficiency anaemia no seroreactivity or reactivity against only one glycan components was more commonly seen (Table 4). Out of the CD patients with multiple antibodies positivity, 65.4% were diagnosed because of malabsorption, which was significantly higher than in CD patients with another serotype group (0 = 26.9%, or 1 = 34.8%, *P* = 0.019) (Figure 2).

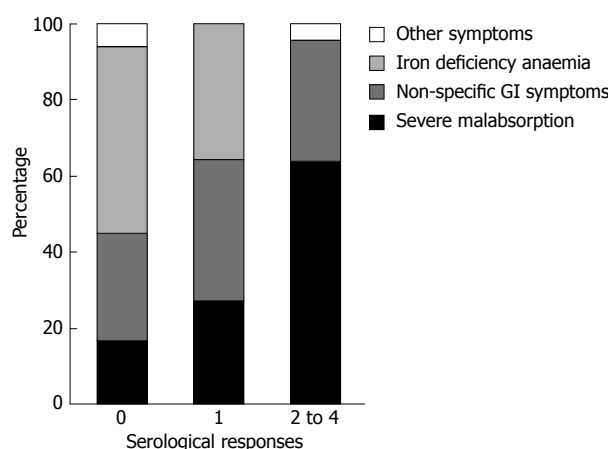
**Correlation between anti-glycan and anti-OMP antibodies and TGA or EMA**

A significant correlation was found between anti-glycan and TGA levels (*P*<sub>gASCA</sub> < 0.001, *R* = 0.39; *P*<sub>AMCA</sub> = 0.01, *R* = 0.28; *P*<sub>ALCA</sub> = 0.006, *R* = 0.23; *P*<sub>ACCA</sub> < 0.0001, *R* =

**Table 3** Association between the titer of anti-microbial antibodies and the leading clinical symptoms at the time of celiac disease presentation (in Group 1, *n* = 78<sup>1</sup>)

	Malabsorption	Non-specific gastrointestinal	Anaemia
<i>n</i>	32	34	9
gASCA IgG	54.8 (16.4-99.4) <sup>b</sup>	21.3 (7.1-76.5)	12.2 (8.8-36.7)
AMCA IgG	90.3 (59.5-115.9) <sup>b</sup>	73.8 (52.3-104.4) <sup>a</sup>	44.1 (25.1-55.0)
ALCA IgG	20.6 (16.7-31.2) <sup>b</sup>	23.7 (16.5-39.1) <sup>a</sup>	12.8 (9.9-15.2)
ACCA IgA	103.8 (53.3-192.1) <sup>b</sup>	57.3(37.4-100.5) <sup>a</sup>	26.8 (12.7-53.6)
OMP IgA	14.8 (5.2-33.2)	15.2 (8.2-24.2)	16.2 (2.9-34.1)

<sup>1</sup>Detailed clinical data were not available for 4 patients, data of 3 patients with other symptoms are not shown; <sup>a</sup>*P* < 0.005, <sup>b</sup>*P* < 0.01, between patients with non-specific gastrointestinal symptoms or malabsorption and anaemia by *t*-test for independent samples with separate variance estimates.



**Figure 2** Clinical presentation of celiac disease according to serological response.

0.53; *P*<sub>antiOMP</sub> = 0.001, *R* = 0.25 by Spearman's rank order correlation). Similarly, a positive association was found between EMA IgA and gASCA (*P* < 0.001), AMCA (*P* < 0.001), ACCA (*P* < 0.0001), or any-glycan (*P* < 0.0001) but not with anti-OMP positivity.

**The effect of strict gluten-free diet on anti-glycan and anti-OMP antibody positivity**

In the group of 30 patients who were evaluated both at diagnosis and following a long term GFD (subgroup of Group CD1), initial positivity for anti-glycan antibodies (gASCA in 12, AMCA in 9, and ACCA in 11 patients) observed at diagnosis was lost after GFD. The titer of each antibody decreased significantly after adherence to GFD (*P* < 0.001 for each). Anti-OMP antibody positivity behaved similarly, with all but one of 14 patients positive at diagnosis becoming negative after GFD. The one patient who did not become negative during a 135 month-long period of GFD did in fact decrease during GFD from 33.2 to a borderline positive value of 25.4 units (a positive result is defined as ≥ 25 units).

The level of the different antibodies was also significantly lower after GFD (*P* < 0.001 for each). Figure 3 shows individual anti-glycan and anti-OMP antibody titers at the time of diagnosis and their changes

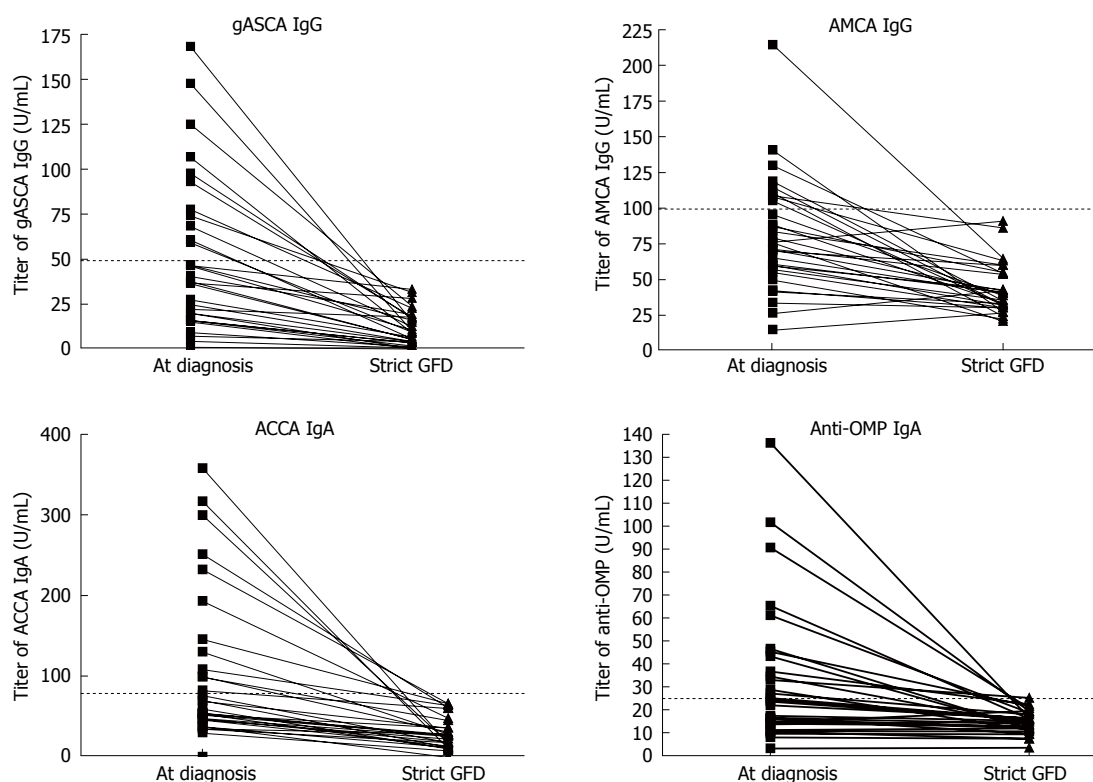


Figure 3 Individual anti-glycan and anti-OMP antibody titers at the time of the diagnosis and their variations after successful adherence of to the gluten-free diet (GFD). Mean follow up period of 49 [10-159] mo ( $n = 30$ ). Dotted lines show cut-off values for positivity.

after successful adherence of to the GFD.

The frequency of antibodies directed against glycans and the mean antibody titers were significantly lower in patients with successful adherence to GFD (Group CD3) than in untreated patients (Group CD1) (Table 2, Figure 1) and did not differ statistically from healthy controls.

#### Prevalence of NOD2/CARD15 mutations and their association with antibody titers and symptoms at presentation

The prevalence of NOD2/CARD15 mutation in CD (19/134, 14.2%) did not differ from that in the control group (16/100, 16%). Additionally, we did not observe any association between symptoms at presentation or anti-glycan antibody positivity and the presence of NOD2/CARD15 variants (data not shown).

## DISCUSSION

This is the first report to investigate the complex associations between a panel of new serological markers, clinical presentation of the disease, and NOD2/CARD15 status in a relatively large cohort of CD patients. Furthermore, direct comparison between the anti-microbial responses in this CD group and our similarly tested previous Crohn's disease cohort<sup>[15]</sup> can be made to add new pieces to the puzzle of the anti-microbial antibody formation.

In this study, we demonstrated that the presence of anti-glycan antibodies (gASCA, ACCA, and AMCA) are associated with CD at the time of diagnosis. However

the prevalence of ALCA and anti-OMP did not differ from the results in the control group. The rate of gASCA positivity (39%) at the time of diagnosis of CD was comparable to the results in CD patients in previous studies<sup>[22-26]</sup>. Based on previous results a sample size of 42-66 celiac patients and controls would have been needed to confirm the above difference with an alpha error of 5% and a statistical power of 95%. In fact, in the present study, for celiac disease at diagnosis the alpha error was 3% and the statistical power 97%.

We could not concur with the findings of Candelli *et al*<sup>[26]</sup> and Barta *et al*<sup>[33]</sup>, which showed significant differences in the prevalence of ASCA IgG between CD and Crohn's diseases. In contrast, no significant difference was noted between the two groups (39% *vs* 50.5%,  $P = 0.091$ ). In the present study, except ALCA, the occurrence of other anti-glycan antibodies and their median titers in CD at diagnosis was also similar to those observed in Crohn's disease<sup>[13,34]</sup> (celiac disease gASCA: 33.1 U/mL, AMCA 79.3 U/mL, ALCA 21.5 U/mL, ACCA 68.4 U/mL *vs* Crohn's disease gASCA: 48.3 U/mL, AMCA 55.5 U/mL, ALCA 25.4 U/mL, ACCA 46.2 U/mL). In addition, the positivity rate for any anti-glycan antibody was also comparable in these patient groups (CD *vs* Crohn's disease: 65.9% *vs* 59.4%,  $P = NS$ ). In addition, sensitivity, specificity, positive and negative likelihood ratios in celiac disease are comparable to that observed in Crohn's disease. Consequently, in patients with gastrointestinal symptoms, the presence of gASCA, AMCA, or ACCA may not only suggest underlying Crohn's disease but may also be associated with untreated CD. At the same time, and based on our

results, ALCA and anti-OMP proved to be specific but relatively non-sensitive markers for Crohn's disease.

Current data advocate that in both CD and Crohn's disease patients have a primary defect in intestinal permeability that is also shared by a subgroup of relatives. In CD, it is also apparent that the exposure to gluten results in mucosal inflammation and the consequent tissue damage further abrogating the primary gut barrier defect, while gluten removal resolves the enhanced intestinal permeability<sup>[35,36]</sup>. These gliadin-induced mechanisms are proposed to be the cause of the anti-microbial antibodies formation in the disease and is strongly supported by the association found between anti-glycan markers and TGA or EMA in the present study and also that the antibody status is substantially altered following the introduction of GFD. gASCA and other positive anti-glycan antibodies were entirely lost in our cohort of CD patients, after strict adherence to long-term GFD. These results are concordant with previous findings<sup>[20,22]</sup>. In the study of Mallant-Hent *et al.*<sup>[24]</sup>, ASCA IgG or IgA positivity disappeared in a substantial number but not the all of the 111 patients on a strict GFD (from 28.8% to 8.1%). A possible explanation for this difference could be that the mean follow up period after GFD was longer in our study [49 (10-159) mo *vs* 33 (range 3-113) mo]. These results suggest that as the period of strict GFD increases, so is the greater disappearance of antibody positivity, which will supposedly lead to entire mucosal healing in the small intestine. The higher prevalence of ASCA in adults compared to children further underlines the important role of long-lasting inflammation and consequently antigen exposure in the formation of anti-microbial antibodies.

In the present study, we also established that the kinetics of antibody disappearance is variably sensitive to the length of GFD. Of the anti-glycan antibodies, AMCA and ACCA declined most rapidly, right after the TGA titer started to diminish. In Group CD2, the prevalence of these antibodies had already changed as compared to Group CD1, from approximately 36% to 11%, while the frequency of gASCA and anti-OMP remained unchanged. Among those CD patients who adopted a strict GFD, the duration of GFD was the shortest in this group. In the group of patients with a successful response to GFD (Group CD3), the frequency of gASCA as well as AMCA and ACCA was also lower. At the same time, the overall frequency of anti-OMP did not change, either in group CD2 or in CD3 (Table 1). We showed however, that the level of anti-OMP clearly declined to normal in 13 of the 14 anti-OMP positive CD patients when specific patients in group CD1 were followed (Figure 2). The explanation of this supposedly inconsistency may be that the mean follow-up in both groups CD2 and CD3 was significantly shorter than in those in group CD1 participating in intra-individual longitudinal monitoring, suggesting that anti-OMP requires the longest time to disappear completely and this occurs long after the normalization of TGA and EMA. The differences in the evolution of anti-OMP and anti-glycan antibodies in IBD has also justified our findings in this patient group<sup>[15]</sup>.

We evaluated the possible relationship between sero-

logical response and the clinical presentation of the disease. Patients with multiple seroreactivity to glycans, more commonly presented with severe malabsorption as compared to those without any reactivity against any glycan at all (63% *vs* 22%,  $P = 0.019$ ), and accounted for 53% of all malabsorption cases. Among the patient groups, the TGA titer reached the highest value (115.9 U/mL *vs* others: 60.9 U/mL,  $P = 0.016$ ) in those presenting with malabsorption, further supporting enhanced intestinal permeability as a likely component involved in antibody formation. It is well known that the intestinal damage is most pronounced in the malabsorption cases and TGA is a good marker for tissue injury<sup>[37]</sup>. We must note however, that the number of subjects in different clinical presentation groups were limited, thus further studies with a larger cohort of CD patients are needed to confirm these findings.

Recent data suggest that the presence of anti-microbial antibodies might be linked to genetic susceptibility. In patients with Crohn's disease an association was found between antimicrobial formation and the carriage of mutations in innate immunity receptor genes (NOD2/CARD15 or toll-like receptor)<sup>[15,20]</sup>. However, in the absence of NOD2 variants in our Crohn's patients' cohort, the gASCA and the any-glycan positivity was also reasonably high (43.5% and 53.7%, respectively). Furthermore, among CD patients in the present study we found that these antibodies occur with the same frequency and magnitude as in patients with Crohn's disease, albeit the occurrence of NOD2/CARD15 mutation was significantly lower. These findings - alongside with the fact that there is no association between TLR4 variants and CD<sup>[38,39]</sup> - do not support the primary role of genetic predisposition in antibody formation. Nevertheless, the presence of NOD2/CARD15 was associated with an increased antibody formation in Crohn's disease and an apparent link was also reported between increased permeability and NOD2/CARD15 3020insC mutation<sup>[40]</sup>. We did not observe any association between anti-glycan antibody positivity and the presence of NOD2/CARD15 variants in CD. However, the limited number of subjects carrying NOD2/CARD15 mutations might not have allowed us to recognize significant differences in serological response in this patients group. An inheritable trait of anti-microbial antibody formation is unlikely in CD, since we did not find a higher prevalence of ASCA (9.1% *vs* 14%) and anti-OMP (12.1% *vs* 20%) as compared to the controls in the 66 unaffected, first-degree relatives (siblings) of this cohort.

On the basis of significant similarity in the qualitative and quantitative serological response in the two patients' groups, we hypothesize a similar mechanism for the formation of the anti-microbial antibody formation in both celiac disease and Crohn's disease. The presence of serological response might be the reflection of the sustained exposure to the constituent of the gut microflora due to the enhanced bacterial translocation. The known predisposing factors for bacterial translocation, such as bacterial overgrowth in the small bowel (secondary to intestinal dysmotility)<sup>[41-43]</sup>, the damage to the integrity of the gut

mucosa (secondary to alterations of the local intestinal microvasculature)<sup>[44,45]</sup>, which results in reduced oxygen delivery and an increased formation in oxygen radicals<sup>[46]</sup> as well as the upregulation of the proinflammatory cytokines, such as tumor necrosis factor  $\alpha$ , interleukin-17 or interferon gamma in active lesions<sup>[47]</sup>, and the defective mucosal immunological defense<sup>[21,48]</sup> are all typical features in both clinical conditions. The significance of the enhanced bacterial translocation out of the small bowel in the anti-microbial antibody formation is further supported by the fact that the presence of the serological response among patients with Crohn's disease is mainly characteristic for those with complicated (stricturing or penetrating) small bowel involvement and is rarely observed in the isolated colonic disease or in patients with ulcerative colitis. At the same time, the recovered gut barrier function protects against the invasion of microbes or their components leading to the cessation of anti-microbial antibody formation. In CD, this process may be justified by the observation that the serological response is a temporary phenomenon. As a result of the discontinuation of gliadin exposure and the subsequent mucosal healing, the antibodies disappear completely. Confirming this aspect of our hypothesis is much more complicated in Crohn's disease. First of all, the pathogenetic processes are not only multifaceted but also less characterized as compared to CD. The complete elimination of the causative agents is not possible. Moreover, no such reliable serological markers are available reflecting the extent of gut inflammation as TGA and anti-actin IgA antibodies in CD. Finally, in terms of the complete loss of microbial seroreactivity, the long-lasting complete remission (without mucosal inflammation) is mandatory but rarely reached in patients with Crohn's disease as compared to CD patients adhering to a strict GFD. In this point of view, findings reporting a lack of solid correlation between disease activity and the presence or the magnitude of seroreactivity<sup>[17,49]</sup> in Crohn's disease can not be in opposition to our hypothesis any more. The advent of the new biological treatments might answer this unresolved question, since the complete mucosal healing in Crohn's disease can be achieved with this therapy in a greater proportion of cases than with classical drugs. At this moment, however, no data from prospective studies are available addressing the effect of the biological therapy on antibody stability. Our data also call for additional basic research to explore the exact mechanism of immune responses to commensal enteric bacteria as well as the possible clinical significance of the bacterial translocation in the pathogenesis or the complications of these diseases as it is well established in other clinical conditions such as liver cirrhosis, acute pancreatitis or sepsis<sup>[50]</sup>.

In conclusion, our results suggest that ASCA and other anti-glycan antibodies may be considered as additional markers for CD and adherence to a GFD. Furthermore, the presence and the magnitude of response to microbial components is associated with a more severe clinical course but not with mutations in NOD2/CARD15. This seroreactivity may be the consequence of the enhanced bacterial translocation through the impaired small bowel mucosa.

## COMMENTS

### Background

Anti-microbial antibody formation has been reported in celiac disease. Relatively high positivity rates were observed for the conventional antibodies, for example, anti-Saccharomyces cerevisiae (ASCA), anti-OmpW, and anti-I2, and they were known to decrease after a successful gluten free diet.

### Research frontiers

Newly discovered inflammatory bowel disease-associated antibodies (including anti-glycan antibodies and anti-OMP) may also be of importance in celiac disease, however, not studied thus far in the published literature. The presence of anti-microbial antibodies in relation to clinical presentation of the disease and NOD2/CARD15 mutations was also not investigated.

### Innovations and breakthroughs

Anti-glycan antibody positivity is a common feature of celiac disease at the time of diagnosis and is lost after long-term gluten-free diet. The positivity rate and titers at diagnosis are as high as observed in Crohn's disease. The presence of anti-glycan antibodies is associated with the presenting symptoms, especially with severe malabsorption but not with mutations in NOD2/CARD15. We did not find a higher prevalence of anti-microbial antibodies in the unaffected, first-degree relatives of this patient cohort.

### Applications

The data may add new pieces to the puzzle of the anti-microbial antibody formation and also assist to re-evaluate recently proposed mechanisms. Serological response to various microbial antigens might be considered a universal marker of the enhanced translocation of the gut microflora through the impaired small bowel mucosa both in celiac and Crohn's disease patients.

### Terminology

Serology markers: anti-endomysial antibodies, synthetic deamidated gliadin peptides, antibodies against microbial antigens such as cell wall component of Saccharomyces cerevisiae, outer membrane porin C transport protein of the Escherichia coli (OmpC) or the Pseudomonas fluorescens associated protein (I2), anti-glycan antibodies: glycan-ASCA (gASCA), anti-mannobioside (AMCA), anti-laminaribioside (ALCA), anti-chitobioside (ACCA).

### Peer review

Papp *et al* studied the prevalence of antimicrobial antibodies in celiac disease patients. The most relevant finding is that anti-glycan antibody titers were associated with symptoms at presentation and their positivity was lost after longstanding gluten free-diet as well as patients with multiple anti-glycan antibodies at diagnosis had more frequently severe malabsorption.

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