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Evidence of Crohn's disease-related anti-glycoprotein 2 antibodies in patients with celiac disease

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Abstract

Background: Autoantibodies to exocrine-pancreatic glycoprotein 2 (anti-GP2) are Crohn's disease (CD) markers. However, CD-specific antibodies have also been found in celiac-disease (CeD) patients, in which type 1 diabetes-specific autoantibodies against endocrine pancreatic targets can be present. We investigated whether anti-GP2

are also present in CeD, a disease like CD which is also characterised by intestinal mucosal inflammation with barrier impairment.

Methods: Antibodies against GP2, tissue transglutaminase (tTG), deamidated gliadin (dGD), glutamic decarboxylase (GAD), and islet antigen-2 (IA2) were tested in sera from 73 CD patients, 90 blood donors (BD), and 79 (58 de novo) CeD patients (2 consecutive sera were available from 40 patients).

Results: IgA and/or IgG anti-GP2 were found in 15/79 (19.0%) CeD patients on at least one occasion, in 25/73 (34.2%) CD patients, and in 4/90 (4.4%) BD (CeD vs. CD, $p=0.042$; BD vs. CeD and CD, $p<0.001$, respectively). Amongst the 58 de novo CeD patients, anti-GP2 IgA and/or IgG were present in 11 (19.0%). Anti-GP2 IgA was significantly less prevalent in CeD compared with CD ($p=0.004$). Anti-GP2 IgA and IgG in CD patients demonstrated a significantly higher median level compared to patients with CeD ($p<0.001$, $p=0.008$, respectively). IgA anti-GP2 levels correlated significantly with IgA anti-tTG and anti-dGD levels in CeD Spearman's coefficient of rank correlation (ρ)=0.42, confidence interval (CI): 0.26–0.56, $p<0.001$; $\rho=0.54$, CI 0.39–0.65, $p<0.001$, respectively.

Conclusions: The presence of anti-GP2 in CeD patients supports the notion that loss of tolerance to GP2 can probably be a manifestation of an autoinflammatory process in this intestinal disorder.

Keywords: celiac disease; Crohn's disease; glycoprotein GP2; intestinal autoimmunity.

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Introduction

Glycoprotein 2 (GP2) has been identified as the main autoantigenic target of Crohn's disease (CD)-specific pancreatic antibodies (PAB) [1–3]. Apart from its previously assumed restricted location in the pancreas, recent data have demonstrated that GP2 is also a constituent of

microfold (M) cells of the follicle-associated epithelium, which appears to have an antimicrobial effect, like its renal homolog uromodulin (Tamm-Horsfall protein) [4–6]. Additionally, emerging evidence indicates that GP2 is over-expressed at the site of intestinal inflammation in patients with CD, and that this molecule modulates innate and adaptive immune responses [1, 7, 8]. Loss of tolerance to GP2 has been reported in up to 30% of CD patients compared to approximately 8%–10% of patients with ulcerative colitis (UC), the other main inflammatory bowel disease (IBD) [9–11]. The clinical significance of these autoantibodies has been assessed and seropositivity for anti-GP2 antibodies appears to identify CD patients with ileocolonic location, stenosing behaviour, and early disease onset [6, 12–16].

Of interest to the present study, CD-related PAB have been detected in patients with celiac disease (CeD), a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals [17, 18]. Exposure to gluten in these patients triggers inflammatory processes leading to a variable degree of intestinal damage which is reversible with the initiation of gluten-free diet (GFD) [18]. The destructive mucosal changes detected in duodenal and jejunal biopsies lead to villous atrophy with hyperplasia of the crypts, a raised intraepithelial lymphocyte count, and an impairment of the intestinal barrier, a clinical complication also seen in patients with IBD [19, 20]. In contrast to the mucosal inflammatory changes in CeD, the transmural inflammation in CD covers all layers of the bowel wall and adventitia and can occur throughout the intestinal tract [3, 21]. Severe tissue lesions, such as fissures, abscesses, strictures, and fistulas, can develop in the course of CD [3].

The immunopathogenesis of IBD as well as that of CeD are poorly understood [22–24]. Antigen-driven mechanisms of immunological breakdown operate in both conditions, but it is still unclear whether the loss of tolerance to GP2 can also be seen in a sub-group of patients with CeD.

In the present study, we considered that it could be of interest to assess PAB-specific anti-GP2 antibody reactivity in patients with CeD, especially in those with de novo manifested disease before initiation of GFD. To this end, we studied IgA and IgG anti-GP2 in well-defined cohorts of serum samples from patients with CeD collected at two different time points over the course of the disease. We then compared the evidence of GP2 autoreactivity in CeD with that in a large number of patients with CD and healthy blood donors. Finally, we assessed the magnitude of PAB-related anti-GP2 antibodies in comparison to that of antibodies against the endocrine pancreas, such as those against the type 1 diabetes (T1D)-specific glutamic acid decarboxylase

(GAD) and islet antigen-2 (IA2) autoantigens, as well as the CeD-related human tissue transglutaminase (tTG) autoantigen and deamidated gliadin (dGD).

Materials and methods

Subjects

Three cohorts of patients with CeD were enrolled in the study including: 1) 42 patients with CeD (including 21 with de novo CeD) from the Department of Laboratory Medicine, University Hospitals Leuven, Catholic University of Leuven; 2) six patients with de novo CeD from the Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, Magdeburg; and 3) 31 with de novo CeD from the Department of Internal Medicine, Attikon Hospital, University of Athens, respectively. Overall, 58 of the 79 (73.4%) CeD patients had de novo manifested CeD. Demographics and laboratory correlates of the CeD patients including Marsh histopathological classification is given in Table 1.

Seventy-three patients with CD from Leuven Hospital and 90 control sera from healthy blood donors (BD) were also assessed. Clinical diagnoses were based upon standard clinical, radiological, endoscopic and histological criteria.

The study was approved by the Local Ethics Committee and complies with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals. Aliquots of the sera stored at -20°C were used to detect antibody reactivity.

Detection of anti-GP2 antibodies by ELISA

Anti-GP2 IgG and IgA antibodies were assessed in serum samples of patients and controls by a commercially available enzyme-linked immunosorbent assay (ELISA) (GA Generic Assays, Dahlewitz/Berlin, Germany), according to the manufacturer's instructions [9]. These assays employ recombinant human GP2 expressed in *Spodoptera frugiperda* 9 cells, as described elsewhere [9]. Optical density was read in a microplate reader at 450 nm and results expressed as arbitrary units (U/mL). The cut-off for positivity at 20 U/mL was used for both anti-GP2 IgA and IgG antibody evaluation in accordance with the recommendations of the manufacturer. The functional assay sensitivity representing the lowest antibody concentration with a coefficient of variation of smaller than 20% was determined at 2.4 U/mL and 1.8 U/mL for anti-GP2 IgG and anti-GP2 IgA, respectively.

ELISA for the detection of CeD-specific antibodies

Serum IgA and IgG antibodies to human recombinant tTG (anti-tTG) and dGD (anti-dGD) were determined by ELISA according to the instructions of the manufacturer (GA Generic Assays GmbH) [27]. The optical density was read in a microplate reader at 450 nm and results expressed as arbitrary units (U/mL). The cut-off for positivity at 10 U/mL in accordance with the recommendations of the manufacturer was used for these assays. The functional assay sensitivity

Table 1 Patients' and blood donors' (BD) characteristics.

	CeD	CD	BD
n	79 ^a	73	90
Mean age (IQR)	24.0 (12.0–42.0)	36.5 (30.0–43.0)	29.5 (22.0–41.8)
Gender f/m	55/24	38/35	33/57
De novo disease	58	na	na
2nd sample	40 ^b	0	0
Marsh classification			
1	1		
2	1		
3a	12		
3b	28		
3c	20		
Not available	17		

^a Nine of 42 patients with celiac disease (CD) referred to Leuven Hospital were from other institutions. For all 9 patients, anti-tissue transglutaminase (tTG) antibody levels were >10 times the upper limit of normal, and for 8 of them anti-tTG antibody levels were >13 times the upper limit of normal (Genesis Diagnostics Ltd, Littelport, UK). The likelihood ratio for CeD is 117 and 319 for anti-tTG levels >10 times and >13 times the upper limit of normal, respectively [25]. Furthermore, all 9 patients demonstrated double positivity of antibodies to tTG and deamidated gliadin (dGD), which is highly indicative for CeD [26]. One of these patients had dermatitis herpetiformis and 1 patient had type 1 diabetes with transient elevation of anti-tTG as well as anti-dGD IgA and IgG (no biopsy data available and no GFD). ^bThe median time delay between the first and second sample analyzed was 9 months (minimum 3 months, 25 percentile 5 months, 75th percentile 22 months and maximum 66 months). One patient reintroduced gluten (with concomitant increase in IgA anti-tTG, whereas another patient did not follow a strict diet (with increases in IgA anti-tTG levels). CD, Crohn's disease; IQR, interquartile range; na, not available.

[28] was determined at 2 U/mL and 3 U/mL for anti-tTG and anti-dGD IgA, respectively, and 3 U/mL and 2 U/mL for IgG to tTG and dGD, respectively.

ELISA for the detection of T1D-specific antibodies

Anti-GAD (anti-GAD) and anti-IA2 antibodies (anti-IA2) were evaluated by ELISA according to the instructions of the manufacturer (Medipan GmbH, Dahlewitz/Berlin, Germany). Briefly, ELISA plates coated with recombinant GAD or IA2 (islet cell antigen 512) were incubated with neat serum samples over night at 2–8 °C. The assay system employs the ability of anti-GAD and anti-IA2 antibodies acting bivalently and forming a bridge between the immobilised autoantigens and their liquid-phase biotinylated variants. The biotinylated autoantigens bound by the respective serum antibodies allow antibody quantification through interaction with streptavidin-peroxidase and the corresponding colorogenic substrate. Absorbance values (at 450 nm) were expressed as international units (IU) calibrated against World Health Organization (WHO) reference preparation NIBSC 97/550 for anti-GAD and anti-IA2 antibodies, respectively.

The cut-offs for positivity at 5 IU/mL for anti-GAD and at 10 IU/mL for anti-IA2 antibodies were used in accordance with the recommendations of the manufacturer. The functional assay sensitivity was determined as 4 IU/mL and 0.8 IU/mL for anti-GAD and anti-IA2 antibodies, respectively.

Statistical analysis

A Kolmogorov-Smirnov test was used to analyse the data for normality. The measured values were expressed as medians with 95% confidential

intervals (CI). The two-tailed, non-parametric Mann-Whitney and Kruskal-Wallis tests were used to test for statistically significant differences of independent samples in two or more groups, respectively. The non-parametric Wilcoxon test was employed to test paired samples.

Spearman's rank correlation test was applied for within group comparison. Comparison of prevalence rates between groups was performed by two-tailed Fisher's exact test. p-Values of <0.05 were considered significant. Calculations were performed using Medcalc statistical software (Medcalc, Mariakerke, Belgium).

Results

Prevalence of autoantibodies in gastrointestinal patients and controls

Anti-GP2 IgG positivity was not significantly different in patients with CeD compared to CD patients (9/79, 11.4% vs. 8/73, 11.0%; $p>0.05$) whereas anti-GP2 IgA positivity was (6/79, 7.6% vs. 19/73, 26.0%; $p=0.004$) (Table 2). Overall, IgA and/or IgG anti-GP2 reactivity was present in 15/79 (19.0%) patients with CeD, which was also significantly less prevalent compared to patients with CD (25/73, 34.2%, $p=0.042$). Both CeD and CD patient groups demonstrated a significantly higher prevalence of IgA and/or IgG anti-GP2 reactivity than BD (4/90, 4.4%; $p=0.003$, $p<0.001$, respectively). Amongst the 58 de novo CeD, 11 (19.0%) had IgA and/or IgG anti-GP2 responses, respectively. The prevalences of IgA and/or IgG anti-GP2 seropositivity did not

Table 2 Prevalence of autoantibodies to glycoprotein 2 (anti-GP2), glutamic acid decarboxylase (anti-GAD), islet cell antigen 2 (anti-IA2), tissue transglutaminase (anti-tTG), and deamidated gliadin (anti-dGD) in 79 celiac disease (CeD) patients (*at least once in 40 patients with two consecutive samples), 73 Crohn's disease (CD) patients, and 90 blood donors (BD) detected by commercial enzyme-linked immunosorbent assay (ELISA).

	IgG anti-GP2, %	IgA anti-GP2, %	IgG/IgA anti-GP2, %	Anti-GAD, %	Anti-IA2, %	IgA anti-tTG, %	IgG anti-tTG, %	IgA anti-dGD, %	IgG anti-dGD, %
CeD n=79 ^a	9 (11.4)	6 (7.6)	15 (19.0)	18 (22.8)	5 (6.3)	76 (96.2)	76 (96.2)	64 (81.0)	69 (87.3)
CD n=73	8 (11.0)	19 (26.0)	25 (34.2)	0 (0.0)	1 (1.4)	0 (0.0)	4 (5.5)	0 (0.0)	1 (1.4)
BD n=90	2 (2.2)	2 (2.2)	4 (4.4)	2 (2.2)	1 (1.1)	nd	nd	nd	nd

differ between the two largest cohorts from Leuven and Athens of de novo CeD patients (5/21 vs. 4/31, $p>0.05$).

As expected, CeD-specific antibodies such as IgA and IgG anti-tTG and anti-dGD demonstrated significantly higher prevalences in patients with CeD compared to those with CD (Table 2, $p<0.001$, respectively).

Concentration of autoantibodies in gastrointestinal patients and controls

IgG and IgA anti-GP2 antibody levels differed significantly amongst patients with CD, CeD, and BD (Kruskal-Wallis test, $p<0.001$; Figure 1). Anti-GP2 IgA in CD patients demonstrated a significantly higher median level compared to patients with CeD [8.0 U/mL, interquartile range (IQR): 4.7–17.1 vs. 4.6 U/mL, IQR: 2.0–7.7; $p<0.001$]. There was also a significantly elevated concentration of anti-GP2 IgG antibodies in patients with CD compared to patients with CeD (8.0 U/mL, IQR: 5.3–11.3 vs. 5.6 U/mL, IQR: 2.5–10.2; $p=0.008$).

The concentration of anti-GAD in patients with CD, CeD, and BD was also significantly different (Kruskal-Wallis

test, $p<0.001$; Figure 2A). In contrast to anti-GP2, anti-GAD levels were significantly higher in patients with CeD compared to those with CD ($p<0.001$). Anti-IA2 also demonstrated different concentrations in the respective groups ($p<0.001$; Figure 2B), whereas its level was significantly higher in patients with CeD compared with CD ($p=0.004$).

In patients with CeD, IgA anti-GP2 levels correlated significantly with IgA anti-tTG and anti-dGD levels [Spearman's coefficient of rank correlation (ρ)=0.42, CI 0.26–0.56, $p<0.001$; $\rho=0.54$, CI 0.39–0.65, $p<0.001$, respectively; Figure 3]. In contrast, anti-GP2 IgG did not correlate with IgG anti-tTG or with anti-dGD antibodies ($p>0.05$).

CeD-, CD-, and T1D-specific antibodies in follow-up samples of CeD patients

Anti-tTG IgA and IgG antibody levels as well as their corresponding prevalences were significantly higher in the first of the two CeD follow-up samples (Table 3, $p<0.001$, respectively). In particular, the median concentrations of anti-tTG IgA and IgG in the first and second samples were as follows: 143.4 U/mL, IQR: 76.4–320.0 vs. 8.8 U/mL, IQR:

Figure 1 IgG and IgA to glycoprotein 2 (anti-GP2) in 119 samples of patients with celiac disease (CeD), 73 samples of patients with Crohn's disease (CD), and 90 blood donor samples (BD) detected by commercial enzyme-linked immunosorbent assay (ELISA). Data are displayed in Box-and-Whisker plots with far out values, defined as values that are smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range, displayed as solid triangles.

Figure 2 Autoantibodies to glutamic acid decarboxylase (anti-GAD) and insel cell antigen 2 (anti-IA2) in 119 samples from 79 patients with celiac disease (CeD), 73 samples of patients with Crohn's disease (CD), and 90 blood donor samples (BD) detected by commercial enzyme-linked immunosorbent assay (ELISA).

Data are displayed in Box-and-Whisker plots with far out values, defined as values that are smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range, displayed as solid triangles.

5.5–28.8 and 79.7 U/mL, IQR: 37.0–201.4 vs. 10.8 U/mL, IQR: 5.5–30.3; respectively, Figure 4A, B). The median concentrations of anti-dGD IgA and IgG antibodies were also higher in the first compared to the second CeD sample ($p < 0.001$, respectively): Anti-dGD IgA were 17.7 U/mL, IQR: 8.8–51.2 vs. 5.3 U/mL, IQR: 2.4–9.0 and anti-dGD IgG were 31.7 U/mL, IQR: 11.1–90.6 vs. 6.5 U/mL, IQR: 3.1–20.2. Prevalences of anti-dGD IgA and IgG were also significantly higher in the first compared to the second sample of the 40 CeD patients with a follow-up sample (Table 3, $p < 0.001$, $p = 0.001$, respectively).

Although there was not a significantly different prevalence for anti-GP2 IgA and/or IgG in the follow-up samples, IgA anti-GP2 levels did show a significant reduction in the second sample (4.6 U/mL, IQR: 2.4–8.9 vs. 2.7 U/mL, IQR: 1.1–5.1; $p < 0.001$). There was also a significantly diminished anti-GP2 IgG antibody reactivity in the second sample compared to the first (7.2 U/mL, IQR: 2.4–13.8 vs.

4.7 U/mL, IQR: 1.7–7.0; $p = 0.033$) (Figure 4 C, D). Interestingly, whereas the prevalence of anti-GP2 IgA and/or IgG was still not significantly different in the first samples of patients with CeD from that in patients with CD, the 40 second samples of CeD patients, mainly under GFD, demonstrated a significant lower prevalence thereof (3/40, 8.5% vs. 25/73, 34.2%; $p = 0.001$). Furthermore, the prevalence of anti-GP2 IgA and/or IgG in the second samples did not differ significantly from that in BD ($p > 0.05$), whereas the prevalence of these antibodies in the first samples did (8/40, 20.0% vs. 4/90, 4.4%, $p = 0.018$).

In contrast, the median of anti-GAD and anti-IA2 levels as well as their corresponding prevalences were not different amongst follow-up samples ($p > 0.05$, respectively).

Amongst the 40 CeD with follow-up serum samples, 37 (92.5%) and 34 (85.0%) have shown a decline of IgA and IgG anti-tTG antibodies in the second sample, respectively (Figure 4). All of the three CeD patients with rising

Figure 3 Correlation of anti-glycoprotein 2 (GP2) IgA with anti-tissue transglutaminase (tTG) and deamidated gliadin IgA concentrations detected by commercial enzyme-linked immunosorbent assay (ELISA) in 79 celiac disease patients (40 patients with two consecutive samples).

Spearman's coefficient of rank correlation (ρ) = 0.42, $\rho = 0.54$; $p < 0.001$, respectively.

Table 3 Prevalence of autoantibodies to glycoprotein 2 (anti-GP2), glutamic acid decarboxylase (anti-GAD), insel cell antigen 2 (anti-IA2), tissue transglutaminase (anti-tTG), and deamidated gliadin (anti-dGD) in 40 celiac disease (CeD) patients with two consecutive samples detected by commercial enzyme-linked immunosorbent assay (ELISA).

CeD n=40	IgG anti-GP2, %	IgA anti-GP2, %	IgG/IgA anti-GP2, %	Anti-GAD, %	Anti-IA2, %	IgA anti-tTG, %	IgG anti-tTG, %	IgA anti-dGD, %	IgG anti-dGD, %
1st samples	6 (15.0)	2 (5.0)	8 (20.0)	8 (20.0)	3 (7.5)	38 (95.0)	38 (95.0)	27 (67.5)	31 (77.5)
2nd samples	2 (5.0)	1 (2.5)	3 (7.5)	9 (22.5)	3 (7.5)	17 (42.5)	21 (52.5)	7 (17.5)	16 (40.0)

IgA anti-tTG levels demonstrated an increase of more than 20% compared to the first value. Of those three, one patient suffered from T1D and had CeD since 2 years under a GFD. The rising CeD-specific antibodies in this patient were due to the introduction of gluten into the diet. The second CeD patient of this group developed CeD and did not follow a strict GFD. The third CeD patient presented with de novo CeD.

Discussion

Both CD and CeD demonstrate inflammation of the intestinal mucosa. However, the localisation of the intestinal destruction and the pathophysiological mechanisms

responsible for the induction of these diseases are quite distinct [3, 29]. Nevertheless, the inflammatory processes seen in CD and CeD are believed to be exacerbated by or lead to an impairment of the intestinal barrier [30, 31]. Growing evidence has been collected to demonstrate that both clinical entities involve the loss of humoral tolerance to self and microbiota antigens [24, 32–36]. We demonstrated in the past, that loss of tolerance to GP2 antigen, an intestinal glycoprotein found apart from its pancreatic localisation in the intestinal M cells of the follicle-associated epithelium, is a characteristic feature of intestinal destruction in patients with CD [6, 12, 15, 37]. In agreement with previous studies reporting GP2 autoantibody detection in up to 30% of patients with CD, we found anti-GP2 IgG and/or IgA antibody reactivity in 34.2% patients with CD. In contrast to the published data, the number

Figure 4 IgA and IgG anti-tissue transglutaminase (tTG) and anti-glycoprotein 2 (GP2) levels detected by commercial enzyme-linked immunosorbent assay (ELISA) in 40 celiac disease patients with two consecutive samples.

of anti-GP2 IgA positive patients with CD in our series exceeded that of anti-GP2 IgG antibodies [9, 11, 38, 39]. The great majority of our CeD patients (60 out of 62) with a known histopathological assessment had a Marsh scoring of 3 preventing us from further analysis associating the presence of anti-GP2 antibodies with tissue destruction. This issue could be solved in a larger cohort including various Marsh scores.

We postulated that loss of tolerance to the GP2 specifically interacting with the intestinal microbiota [40] may be a generic feature of conditions characterised by an inflammation of the intestinal mucosa, and we assessed this hypothesis by testing patients with CeD. To some extent, our assumption has been a valid one. We have identified patients with CeD, in whom anti-GP2 antibodies were detectable. Our data were obtained testing three different patient cohorts, as well as a significant number of de novo manifested CeD using the same ELISA kits. Thus, we have been able not only to assess the prevalences of anti-GP2 antibodies in de novo CeD versus follow-up serum samples but also to compare GP2-specific responses amongst geographically distinct cohorts from Germany, Belgium and Greece. Our data reached a consensus: IgA anti-GP2 antibodies are present in a considerable number of CeD patients.

The frequency of anti-GP2 IgG seropositivity was not significantly different in the CeD compared to the CD cohort but was higher compared to that in BD. However, the median levels of IgA as well as IgG anti-GP2 were higher in CD patients. This phenomenon could be due to the lower amount of follicle-associated epithelium and, therefore, M cells in the duodenum, the typical site of inflammation during CeD, compared with the ileum being one of the main inflammatory sites in CD [41–43]. We could support this assumption by revealing a significantly higher prevalence of anti-GP2 IgA and/or IgG in the first follow-up samples of patients with CeD containing patients with de novo CeD in contrast to that in BD. The second samples of those patients with CeD mainly under GFD did not show this significant difference.

During the preparation of this manuscript, limited data in the form of a letter to the editor have also been presented suggesting that GP2 is an autoantibody target in some patients with CeD. Bonaci-Nikolic and colleagues reported the occurrence of anti-GP2 in untreated de novo patients with CeD, which points to the possibility of autoreactivity to GP2 during acute CeD inflammation characterised by a complete loss of the architecture of the small bowel in severe cases [17]. Further evidence of loss of tolerance to GP2 in CeD was reported recently demonstrating in particular anti-GP2 IgA in patients with refractory CeD [44].

We measured anti-GAD and anti-IA2 as internal autoantibody controls since these autoantibodies target T1D-related antigens located in the endocrine pancreas while GP2 is an autoantigen of pancreatic autoantibodies against the exocrine pancreas. In contrast to anti-GP2, anti-GAD and anti-IA2 seropositivity in patients with IBD was not significantly different to that in BD.

Interestingly, we could establish a significant correlation between IgA anti-GP2 and IgA anti-tTG as well as anti-dGD levels investigating 119 sera from the 79 patients with CeD. Also, it seems that autoreactivity to GP2 in CeD follows the trend of loss of tolerance to tTG but such an association could not be found for anti-GAD or anti-IA2. After the onset of a GFD, the CeD-typical inflammation largely disappears as did the antibody reactivity to tTG in the majority of clinical cases. Provided that the loss of tolerance to GP2 antigen in CeD behaves in a similar manner to the well-known autoreactivity to tTG, anti-GP2 antibodies may be an element of intestinal inflammation rather than a sole hallmark of CD. Whether removal of the putative causative inflammatory agent in CD could lead to a reduction of anti-GP2 levels as does removal of gluten for anti-tTG in CeD remains to be seen.

Recently, the occurrence of anti-GP2 has been reported in UC patients with an ileal pouch anal anastomosis developing pouchitis after proctocolectomy [45, 46]. Interestingly, such patients with CeD-like complications demonstrated significantly higher anti-GP2 levels than those without. Noteworthy, the association of anti-GP2 levels with disease activity in CD has been discussed, albeit controversially. Clinical remission is not always accompanied by the disappearance of anti-GP2 [11, 47, 48].

Our data show a correlation between IgA anti-tTG and anti-dDG with anti-GP2 IgA. Whether this is a characteristic feature in CeD remains to be confirmed by further studies. Such correlations were not found in patients with CD. The interpretation of these data has to be treated with extreme caution.

In summary, anti-GP2 IgG and IgA can occur during intestinal inflammation in a proportion of patients with CeD. The diagnostic and clinical significance of these findings require external validation and further investigation in a large cohort of prospectively collected serum samples from patients with CeD followed up for a long time.

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References

- Roggenbuck D, Hausdorf G, Martinez-Gamboa L, Reinhold D, Buttner T, Jungblut PR, et al. Identification of GP2, the major zymogen granule membrane glycoprotein, as the autoantigen of pancreatic antibodies in Crohn's disease. *Gut* 2009;58:1620–8.
- Komorowski L, Teegen B, Probst C, Aulinger-Stocker K, Sina C, Fellermann K, et al. Autoantibodies against exocrine pancreas in Crohn's disease are directed against two antigens: the glycoproteins CUZD1 and GP2. *J Crohns Colitis* 2013;7:780–90.
- Laass MW, Roggenbuck D, Conrad K. Diagnosis and classification of Crohn's disease. *Autoimmun Rev* 2014;13:467–71.
- Hase K, Kawano K, Nochi T, Pontes GS, Fukuda S, Ebisawa M, et al. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. *Nature* 2009;462:226–30.
- Terahara K, Yoshida M, Igarashi O, Nochi T, Pontes GS, Hase K, et al. Comprehensive gene expression profiling of Peyer's patch M cells, villous M-like cells, and intestinal epithelial cells. *J Immunol* 2008;180:7840–6.
- Roggenbuck D, Reinhold D, Werner L, Schierack P, Bogdanos DP, Conrad K. Glycoprotein 2 antibodies in Crohn's disease. *Adv Clin Chem* 2013;60:187–208.
- Werner L, Paclik D, Fritz C, Reinhold D, Roggenbuck D, Sturm A. Identification of pancreatic Glycoprotein 2 as an endogenous immunomodulator of innate and adaptive immune responses. *J Immunol* 2012;189:2774–83.
- Holz MA, Hofer J, Kovarik JJ, Roggenbuck D, Reinhold D, Goihl A, et al. The zymogen granule protein 2 (GP2) binds to scavenger receptor expressed on endothelial cells I (SREC-I). *Cell Immunol* 2011;267:88–93.
- Roggenbuck D, Reinhold D, Wex T, Goihl A, von Arnim U, Malfertheiner P, et al. Autoantibodies to GP2, the major zymogen granule membrane glycoprotein, are new markers in Crohn's disease. *Clin Chim Acta* 2011;412:718–24.
- Bogdanos DP, Rigopoulou EI, Smyk DS, Roggenbuck D, Reinhold D, Forbes A, et al. Diagnostic value, clinical utility and pathogenic significance of reactivity to the molecular targets of Crohn's disease specific-pancreatic autoantibodies. *Autoimmun Rev* 2011;11:143–8.
- Op de Beeck K, Vermeire S, Rutgeerts P, Bossuyt X. Antibodies to GP2, the major zymogen granule membrane glycoprotein, in inflammatory bowel diseases. *Gut* 2012;61:162–4.
- Bogdanos DP, Roggenbuck D, Reinhold D, Wex T, Pavlidis P, von Arnim U, et al. Pancreatic-specific autoantibodies to glycoprotein 2 mirror disease location and behaviour in younger patients with Crohn's disease. *BMC Gastroenterol* 2012;12:102.
- Pavlidis P, Romanidou O, Roggenbuck D, Mytilinaiou M, Al-Sulttan F, Liaskos C, et al. Ileal inflammation may trigger the development of GP2-specific pancreatic autoantibodies in patients with Crohn's disease. *Clin Dev Immunol* 2012;2012:640835.
- Somma V, Ababneh H, Ababneh A, Gatti S, Romagnoli V, Bendia E, et al. The novel Crohn's disease marker anti-GP2 antibody is associated with ileocolonic location of disease. *Gastroenterol Res Pract* 2013;2013:683824.
- Rieder F, Franke A, Dirmeier A, Lopez R, Lang S, Roggenbuck D, et al. Mo1247 serologic anti-GP2 antibodies are associated with strictures and need for surgical resection in Crohn's disease. *Gastroenterology* 2013;144:S617.
- Roggenbuck D, Humbel RL, Reinhold D, Bogdanos DP, Conrad K, Laass MW. Glycoprotein 2 antibodies in inflammatory bowel disease – no association with disease phenotype? *J Pediatr Gastroenterol Nutr* 2012;56:e5.
- Bonaci-Nikolic B, Spuran M, Andrejevic S, Nikolic M. Autoantibodies to GP2, the major zymogen granule membrane glycoprotein, in patients with gluten-sensitive enteropathy: a possible serological trap. *Clin Chim Acta* 2012;413:822–3.
- Ludvigsson JF, Leffler DA, Bai JC, Biagi F, Fasano A, Green PH, et al. The Oslo definitions for coeliac disease and related terms. *Gut* 2013;62:43–52.
- Soderholm JD, Olaison G, Lindberg E, Hannestad U, Vindels A, Tysk C, et al. Different intestinal permeability patterns in relatives and spouses of patients with Crohn's disease: an inherited defect in mucosal defence? *Gut* 1999;44:96–100.
- Bjarnason I. Intestinal permeability. *Gut* 1994;35:S18–22.
- Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007;369:1627–40.
- Rieder F, Lawrance IC, Leite A, Sans M. Predictors of fibrostenotic Crohn's disease. *Inflamm Bowel Dis* 2011;17:2000–7.
- Bardella MT, Elli L, De MS, Floriani I, Torri V, Piodi L. Autoimmune disorders in patients affected by celiac sprue and inflammatory bowel disease. *Ann Med* 2009;41:139–43.
- Basso D, Zambon CF, Plebani M. Inflammatory bowel diseases: from pathogenesis to laboratory testing. *Clin Chem Lab Med* 2014;52:471–81.
- Vermeersch P, Geboes K, Marien G, Hoffman I, Hiele M, Bossuyt X. Diagnostic performance of IgG anti-deamidated gliadin peptide antibody assays is comparable to IgA anti-tTG in celiac disease. *Clin Chim Acta* 2010;411:931–5.
- Vermeersch P, Geboes K, Marien G, Hoffman I, Hiele M, Bossuyt X. Serological diagnosis of celiac disease: comparative analysis of different strategies. *Clin Chim Acta* 2012;413:1761–7.
- Conrad K, Roggenbuck D, Ittenson A, Reinhold D, Buettner T, Laass MW. A new dot immunoassay for simultaneous detection of celiac specific antibodies and IgA-deficiency. *Clin Chem Lab Med* 2011;50:337–43.
- Zöphel K, Wunderlich G, Kotzerke J, von Landenberg P, Roggenbuck D. M22 based (manual) ELISA for TSH-receptor antibody (TRAb) measurement is more sensitive than 2nd generation TRAb assays. *Clin Chim Acta* 2009;403:266.
- Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2002;2:647–55.
- Tibble J, Sigthorsson G, Foster R, Sherwood R, Fagerhol M, Bjarnason I. Faecal calprotectin and faecal occult blood tests in the diagnosis of colorectal carcinoma and adenoma. *Gut* 2001;49:402–8.
- de Kort S, Keszthelyi D, Masclee AA. Leaky gut and diabetes mellitus: what is the link? *Obes Rev* 2011;12:449–58.

32. Bossuyt X. Serologic markers in inflammatory bowel disease. *Clin Chem* 2006;52:171–81.
33. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E. Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 1995;155:5419–26.
34. Baekkeskov S, Kanaani J, Jaume JC, Kash S. Does GAD have a unique role in triggering IDDM? *J Autoimmun* 2000;15:279–86.
35. Conrad K, Schmechta H, Klafki A, Lobeck G, Uhlig HH, Gerdi S, et al. Serological differentiation of inflammatory bowel diseases. *Eur J Gastroenterol Hepatol* 2002;14:129–35.
36. Michielan A, Basso D, Martinato M, Pathak S, Banerjee A, Oliva L, et al. Increased antibody response to microbial antigens in patients with Crohn's disease and their unaffected first-degree relatives. *Dig Liver Dis* 2013;45:894–8.
37. Roggenbuck D, Reinhold D, Schierack P, Bogdanos DP, Conrad K, Laass MW. Crohn's disease specific pancreatic antibodies: clinical and pathophysiological challenges. *Clin Chem Lab Med* 2014;52:483–94.
38. Pavlidis P, Forbes A, Bogdanos DP. Antibodies to glycoprotein 2 (GP2) in patients with inflammatory bowel diseases from UK. *Clin Chim Acta* 2011;412:1163–4.
39. Roggenbuck D, Bogdanos D, Conrad K. Loss of tolerance to one or two major targets in Crohn's disease or just cross-reactivity? *J Crohns Colitis* 2013;7:e273–4.
40. Schierack P, Rödiger S, Kolenda R, Hiemann R, Berger E, Grzymailo K, et al. Species-specific and pathotype-specific binding of bacteria to zymogen granule membrane glycoprotein 2 (GP2). *Gut* [Epub ahead of print 2014 Jul 29]. doi:10.1136/gutjnl-2014-307854 (in press).
41. Colombel JF, Watson AJ, Neurath MF. The 10 remaining mysteries of inflammatory bowel disease. *Gut* 2008;57:429–33.
42. Gullberg E, Soderholm JD. Peyer's patches and M cells as potential sites of the inflammatory onset in Crohn's disease. *Ann N Y Acad Sci* 2006;1072:218–32.
43. Cornes JS. Peyer's patches in the human gut. *Proc R Soc Med* 1965;58:716.
44. Gross S, Bakker SF, van Bodegraven AA, van Hoogstraten IM, Gelderman KA. Increased IgA glycoprotein-2 specific antibody titres in refractory CD. *J Gastrointest Liver Dis* 2014;23:1–7.
45. Werner L, Sturm A, Roggenbuck D, Yahav L, Zion T, Meirowitz E, et al. Antibodies against glycoprotein 2 are novel markers of intestinal inflammation in patients with an ileal pouch. *J Crohns Colitis* 2013;7:e522–32.
46. Werner L, Roggenbuck D, Dotan I. Reply to Dr. Pavlidis et al.'s letter. *J Crohns Colitis* 2013;7:e604–5.
47. Roggenbuck D, Reinhold D, Wex T, von Arnim U, Malfertheiner P, Sturm A, et al. Authors' response: antibodies to GP2, the major zymogen granule membrane glycoprotein, are specific for Crohn's disease and may reflect treatment response. *Gut* 2012;61:164–5.
48. Kovacs M, Lakatos PL, Papp M, Jacobsen S, Nemes E, Polgar M, et al. Pancreatic autoantibodies and autoantibodies against goblet cells in pediatric patients with inflammatory bowel disease (IBD). *J Pediatr Gastroenterol Nutr* 2012;55:429–35.