

Increased IgA Glycoprotein-2 Specific Antibody Titres in Refractory Celiac Disease

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ABSTRACT

Background & Aims: In most cases celiac disease (CD) is successfully treated with a gluten-free diet (GFD). However, some patients become refractory to the GFD. Refractory CD (RCD) patients have an increased risk for developing enteropathy associated T-cell lymphoma and early diagnosis is therefore of importance. Currently, RCD diagnosis relies on endoscopy and adequate serological markers are lacking. Antibodies against glycoprotein-2 (GP2A) were described in Crohn's disease (CrD) and active CD but not in ulcerative colitis (UC), suggesting this is a specific marker for small intestinal lesions.

Methods: Sera obtained from patients visiting our outpatient clinic for routine serological tests for diagnosis and/or follow-up of inflammatory bowel disease (n=78), active CD (n=45), GFD (n=34) and RCD (n=15) were analysed for GP2A titres.

Results: Increased GP2A-IgA levels in CrD and active CD as compared to controls ($p < 0.001$) and lack thereof in UC was confirmed. However, we could not confirm the association with small bowel localization within the CrD patient group. Within CD patients, we demonstrated a significant decrease of GP2A-IgA titres upon a GFD and increased levels in RCD patients as compared to patients on a GFD. Although GP2A-IgA was not associated with the degree of villous atrophy, GP2A-IgA levels were able to distinguish RCD patients from GFD patients (ROC AUC=0.79, $p=0.002$).

Conclusion: Follow-up of GP2A-IgA titres in CD patients on a GFD may help to identify patients at risk for developing RCD.

Key words: Celiac disease – Crohn's disease – ulcerative colitis – refractory celiac disease – glycoprotein-2 antibodies.

Abbreviations: ABSA: anti-bovine serum albumine; ASCA: anti- *Saccharomyces cerevisiae* antigen; AU: artificial units; AUC: area under the curve; CD: celiac disease; CrD: Crohn's disease; EATL: enteropathy associated T-cell lymphoma; EmA: anti-endomysium antibodies; GFD: gluten free diet; GP2A: glycoprotein 2 antibodies; IEL: intraepithelial lymphocytes; RCD: refractory celiac disease; ROC: receiver operator curve; TG2A: transglutaminase-2 antibodies; UC: ulcerative colitis.

INTRODUCTION

Celiac disease (CD) is an autoimmune disease characterized by intestinal mucosal damage as a consequence of a hypersensitivity to dietary gluten peptides. A gluten-free diet (GFD) reduces symptoms in the majority of patients, but a small fraction of CD patients develop refractory CD (RCD) [1]. Refractory CD has the same histological features as active CD, i.e. lymphocytosis

of intraepithelial lymphocytes (IEL), villous atrophy and crypt hyperplasia [2]. Refractory CD type II is characterised by the presence of more than 20% aberrant, surface CD3 negative but cytoplasmic CD3 positive, IELs as measured by flowcytometry. These aberrant T-cells are thought to be the origin of enteropathy associated T-cell lymphomas (EATL), and RCD type II patients have thus an increased risk to develop an EATL. While antibodies against tissue transglutaminase (TG2A) are highly sensitive and specific for the diagnosis of active CD [3], currently there are no biochemical parameters that can aid in the diagnosis of RCD; diagnosis relies on invasive endoscopic procedures.

Auto-antibodies against glycoprotein-2 (GP2A), a protein located in both pancreas and M-cells, were recently found to be elevated in Crohn's disease (CrD), but not in ulcerative colitis

(UC), which makes them promising markers for a differential diagnosis of inflammatory bowel disease [4-7]. Interestingly, patients with ileocolonic CrD had higher GP2A levels than patients with colonic CrD [8]. The induction of auto-antibodies against GP2 may therefore be particularly related to small intestinal damage. Indeed, GP2A titres were also increased in patients with active CD [9]. Since RCD and active CD share pathological features, it is expected that GP2A levels would also be elevated in RCD patients but there are no data yet to support this hypothesis.

Here we investigated whether GP2A-IgA and -IgG were indeed related to small bowel disease in a group of patients with active CD, CrD and UC. Furthermore we investigated whether GP2A titres were associated with the degree of villous atrophy in CD and whether these antibodies could serve as biomarkers for RCD. Finally, we compared GP2A titres with antibodies directed against the food antigens bovine serum albumin (ABSA) and *Saccharomyces cerevisiae* (ASCA) since we recently described these to be elevated in RCD as well [10].

MATERIAL AND METHOD

Crohn's disease and UC patients

Crohn's disease and UC patients diagnosed at our hospital were included in this study. The diagnosis of CrD (n=38) and UC (n=40) was based on standard clinical, endoscopic, histological, and radiographic features [11]. Disease localization was documented according to the Vienna classification [12]. All serum samples were obtained before anti-TNF α therapy was initiated [13]. All patients were treated with standard first line immunosuppressive drugs.

Celiac disease and refractory CD patients

Adult CD and RCD patients (age > 18 years) who visited our centre from 2000 to 2011 were included in this study (see Table I for patient characteristics). In this study RCD type II patients, which are well defined by high frequencies (>20%) of cell surface CD3 negative, CD8 negative, intracellular CD3 positive, aberrant intraepithelial T-cells, were included. Patients were registered as active (i.e. recently diagnosed) CD patients (n=45), as being on a gluten-free diet (GFD; n=34) or as diagnosed with RCD type II (n=15) [14]. The sera of these patients were matched with histological data from biopsies that were taken within 4 weeks before or after the date that the respective serum was taken. The status of the intestinal mucosa was classified following the adjusted Marsh criteria [15]. IgA-deficient patients were excluded. Active CD was diagnosed according to guidelines set for adult CD [16], i.e. if biopsies showed increased numbers of intraepithelial lymphocytes, crypt hyperplasia and villous atrophy together with elevated serum antibodies against transglutaminase-2 (TG2A) and anti-endomysium antibodies (EmA). All active CD patients were prescribed a GFD. Adherence to the GFD was certified by an experienced dietitian and additionally monitored by regular (approx. every 3 months) measurements of serum titres of TG2A. From 34 active CD patients consecutive sera were available after a GFD of at least 6 months and these were included in the GFD group irrespective of serological or histological recovery. Follow-up biopsies were taken as per

local protocol, i.e. approx. one year after initiation of the GFD in order to confirm histological recovery in patients that showed clinical improvement, or if RCD was suspected.

Patients were diagnosed with RCD when malabsorption symptoms and histological abnormalities persisted or recurred despite certified and controlled strict adherence to the GFD. Other intestinal diseases such as irritable bowel syndrome or intestinal infections were excluded.

Controls

The control group consisted of 27 subjects who were admitted to our hospital with complaints of sudden deafness, which could be a clinical sign of an autoimmune disease. However, subsequent measurements of auto-antibodies were negative. In these subjects any other sign of autoimmune disease was lacking. Furthermore, 24 healthy volunteers from our laboratory were included in the control group.

The current study adhered to guidelines set by the institutional ethical committee. Sera used remaining after routine diagnosis were used according to the Human Tissue and Medical Research Code of conduct for responsible use (2011) (<http://www.federa.org/codes-conduct>).

GP2A, ABSA and ASCA ELISA

IgA and IgG antibodies against GP2 were measured using commercially available ELISA kits (Anti-GP2-IgA and AntiGP2-IgG, both from GA Generic Assays, Dahlewitz, Germany) according to the manufacturer's instructions. A cut-off for (clinically relevant) positivity at 20 U/ml was used as advised by the manufacturer. The analytical assay sensitivity of the GP2 tests is 0.49U/ml. *Saccharomyces cerevisiae* antibodies (ASCA) were measured with commercial kits (QUANTA Lite ASCA IgA, INOVA Diagnostics Inc., San Diego, USA) according to the manufacturer's recommendations. A cut-off for positivity at 25 U/ml was used. The analytical assay sensitivity of the ASCA tests is 3U/ml. Antibody titres against bovine serum albumin (ABSA) were determined by an in-house ELISA as described previously [10]. The cut-off for positivity was the level at the 95-percentile of the healthy control group. The analytical assay sensitivity of the ABSA tests is 5AU/ml.

Statistical analyses

Data for all parameters tested were not normally distributed (D'Agostino and Pearson's Omnibus K2 test, Graphpad Prism 5.02 software). GP2A-IgA data are therefore presented as median [5 percentile - 95 percentile]. Differences in antibody titres between the patient groups and the control group were tested with the Mann-Whitney-U test. Differences in the frequencies of positive patients were tested with the Fisher's Exact test. The correlation between GP2A-IgA and -IgG was tested using the Spearman correlation. Associations of GP2A, ABSA and ASCA with villous atrophy were tested using the chi-square test. For this test values of the respective serum marker were plotted against Marsh classification. Subsequently, concentrations of the markers within the groups Marsh 0 - II (no villous atrophy) were compared to the concentrations of groups Marsh IIIa-IIIc (villous atrophy). P-values were calculated from two-tailed tests.

In order to calculate sensitivity/specificity of the serum markers for the indication of villous atrophy, we categorized the serum levels into two categories using the cut-off provided by the manufacturer of the ELISA kits (ASCA, GP2A) or in the case of ABSA, using the 95-percentile of the control group.

In order to further investigate a potential use of GP2A, ABSA and ASCA for the follow-up of CD patients on a gluten-free diet, we tested whether GP2A and other serum markers could indicate RCD. We therefore calculated the ROC curve and the area under the curve (AUC) of each serum marker for distinguishing the RCD patients from GFD patients.

RESULTS

Patients' characteristics are shown in Table I.

GP2A in CrD, UC and active CD

Crohn's disease patients had significantly elevated GP2A-IgA levels when compared to controls (3.4 [0.9-49.1] AU/ml versus 1.3 [0.1-19.2] AU/ml; $p < 0.001$ Mann-Whitney-U), while patients with UC did not (1.8 [0.7-7.8] AU/ml) (Fig. 1a). The fraction of GP2A-IgA positive CrD patients was however only 13.2%, as compared to 28.9% for active CD (7.1 [0.9-126.2] AU/ml). While the median GP2A-IgG levels were not elevated in the UC group, the percentage of GP2A-IgG positive patients (12.5%) was increased compared to the control group (0%). Although active CD patients had slightly, yet significantly, elevated GP2A-IgG levels (6.9 [1.8-19.4] AU/ml) when compared to controls (5.0 [2.1-12.7] AU/ml; $p = 0.03$ Mann-Whitney-U), none of the sera reached levels above the cut-off. In CrD patients GP2A-IgG levels were significantly elevated as well (6.7 [1.5-194.7] AU/ml, $p = 0.02$ Mann-Whitney-U) resulting in a significant increase in the proportion of positive CrD patients ($p < 0.02$ Fisher's Exact; Fig. 1b).

GP2A-IgG and GP2A-IgA were correlated ($r = 0.4$, $p < 0.001$; Spearman correlation) (Fig. 2); nevertheless in subsets of patients differential IgA and IgG responses to GP2 were observed. A fraction of CrD patients (13.2%) had GP2A-IgA

Table I. Patients' characteristics

Group	N	Sex, f % (n)	Age, yrs (SD)	TG2A/EmA, pos % (n)
Control	51	52.9% (27)	43.4 ± 15.0	n.a.
Crohn's disease	38	71.1% (27)	36.4 ± 11.8'	n.a.
Ulcerative colitis	40	52.5% (21)	36.5 ± 9.6'	n.a.
Active CD	45	66.7% (30)	44.4 ± 17.4	100% (45)
CD on a GFD ¹	34	70.6% (24)	48.3 ± 17.2	26.5% (9)
Refractory CD type II	15	53.3% (8)	65.3 ± 5.6'	0.0% (0)

n.a. = not analyzed; ' = p -value < 0.001 when compared to the control group, no statistical testing done on TG2A, CD = celiac disease, GFD = gluten-free diet

Table II. Association of GP2A and localization in Crohn's disease

Vienna classification (localization)	GP2A-IgA (AU/ml)	GP2A-IgG (AU/ml)
Terminal ileum (N=5)	2.4 [1.9-36.2]	6.9 [2.7-232.0]
Colon (N=14)	3.4 [0.9-119.1]	6.1 [1.5-192.7]
Ileocolon (N=19)	3.4 [0.8-45.4]	6.5 [1.9-39.3]
Upper gastrointestinal	n/a	n/a

n/a: Not applicable; data shown as median [P5-P95]

levels above 10 AU/ml and GP2A-IgG levels above 30 AU/ml. Some of the active CD patients (17.8%) had high (> 20 AU/ml) GP2A-IgA levels and low (< 20 AU/ml) GP2A-IgG levels. Only two UC patients could be differentiated from CrD by high levels of GP2A-IgG (> 50 AU/ml) and -IgA levels below the cut-off (5% of UC) (Fig. 2). These data suggest that particularly GP2A-IgA may be associated with small bowel disease. However, when GP2A-IgA and -IgG levels were compared between subgroups of CrD patients categorized according to the Vienna

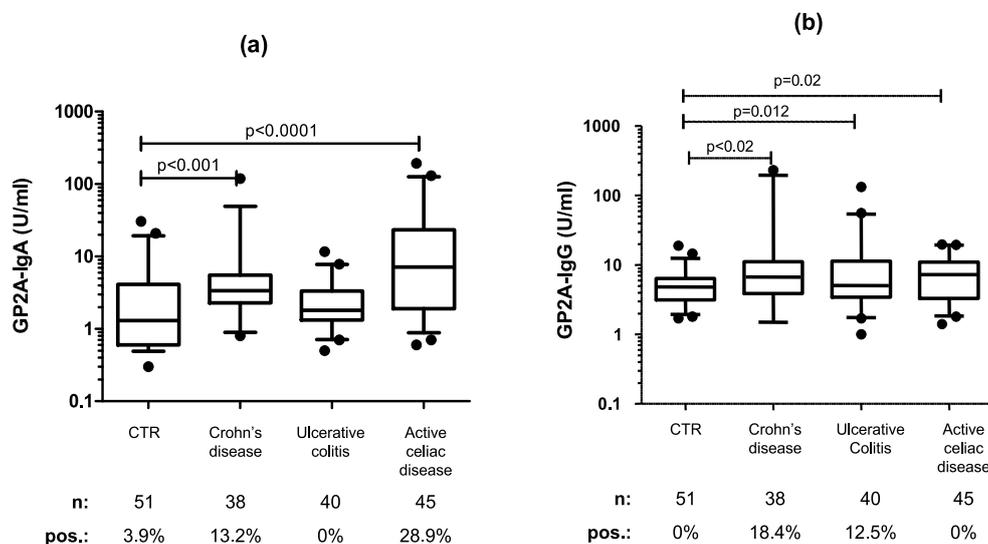


Fig. 1. GP2A in inflammatory bowel disease and active CD. a) IgA, b) IgG.

CTR = control, n = number of patients per group, pos = percentage of patients considered positive. P-values indicate significant differences in GP2A levels (Mann-Whitney-U test). GP2A = Glycoprotein-2 antibodies.

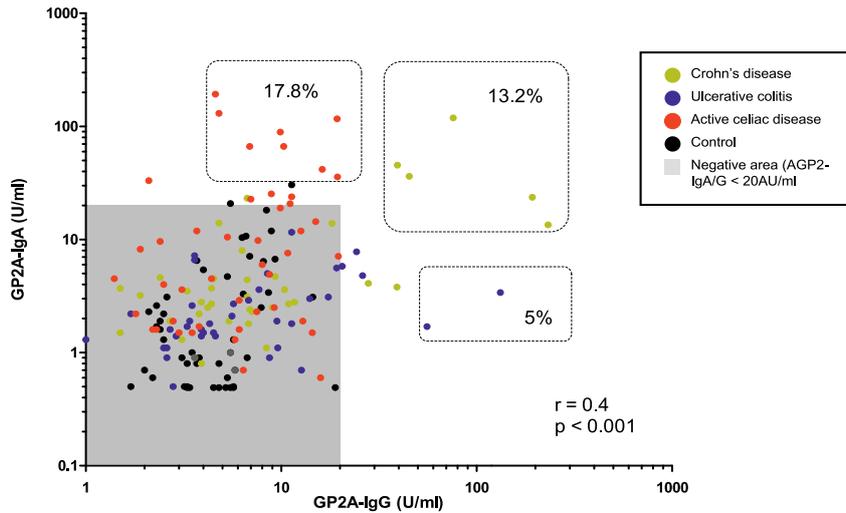


Fig. 2. Correlation of GP2A-IgA and -IgG. Correlation coefficient (r) and probability value (p) were calculated from controls (black symbols) and from patients with CrD (green symbols), UC (blue symbols) and active CD (red symbols) using Pearson's correlation. Negative cases for both markers are located in the shaded area.

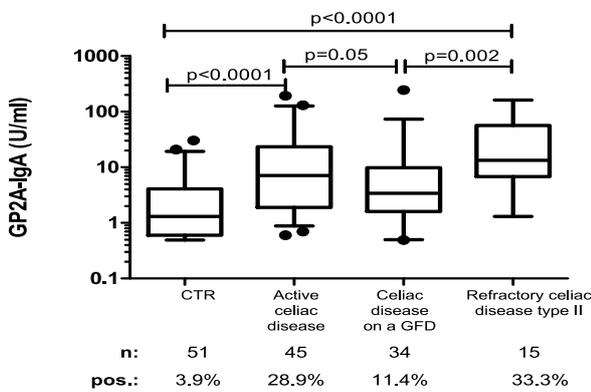


Fig. 3. GP2A-IgA is enhanced in (refractory) CD. CTR = controls, GFD = gluten-free diet, n = number of patients, pos = percentage of patients considered to be positive. P-values indicate significant differences in GP2A levels (Mann-Whitney U-test).

classification of disease localization, no significant association with GP2A-IgA and localisation was observed (Table II).

GP2A in the follow-up of CD patients

As discussed above GP2A-IgA levels were increased in active CD patients as compared to controls (Fig. 1a and Fig 3). With a strict GFD for at least 6 months, titres of GP2A-IgA levels decreased to a median of 3.4 [0.5-73.2] AU/ml ($p = 0.05$, Wilcoxon signed rank) yet the percentage of GP2A-IgA-positive patients did not differ significantly ($p > 0.05$, Fisher's Exact, Fig. 3). Within the RCD group 33.3% of the patients were positive for GP2A-IgA; this patient group also displayed the highest titres (13.3 [1.3-161.6] AU/ml, Fig. 3).

We investigated whether the height of the GP2A antibody response was correlated with the degree of mucosal damage in CD. As shown in Fig. 4, no significant association was found between GP2A-IgA-positivity and the degree of villous atrophy.

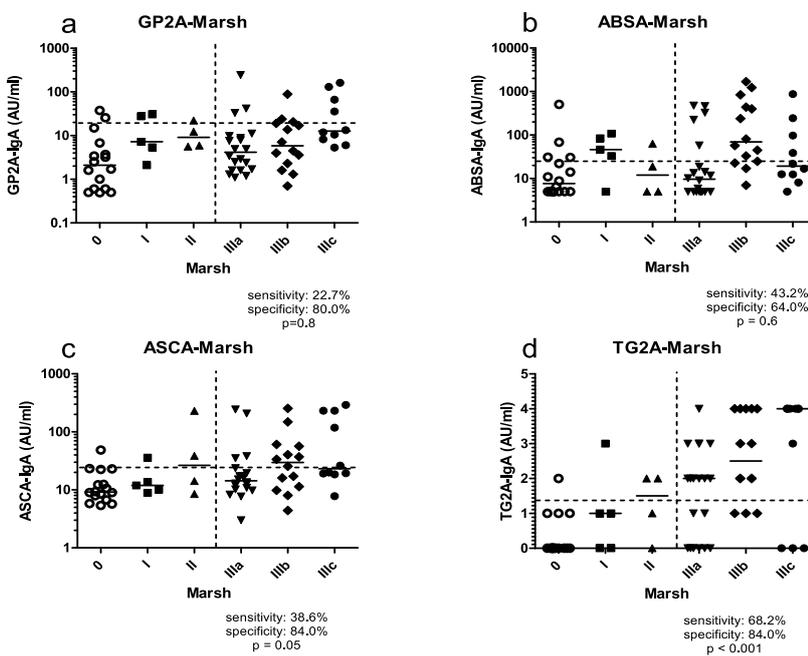


Fig. 4. Association of serum antibodies with mucosal damage in all CD groups. Dotted lines indicate cut-offs used for the calculation of specificities and sensitivities for villous atrophy and for statistical testing. No significant associations between histological damage and the displayed serological parameters could be found (chi-square test). GP2A = Glycoprotein-2 antibodies, ASCA = Saccharomyces cerevisiae antibodies, ABSA = bovine serum albumin antibodies, TG2A = tissue transglutaminase antibodies, TG2A categories: 0 = 0 - 6.9; 1 = 7.0 - 9.9; 2 = 10.0 - 29.9; 3 = 30-99.9; 4 = >100 AU/ml.

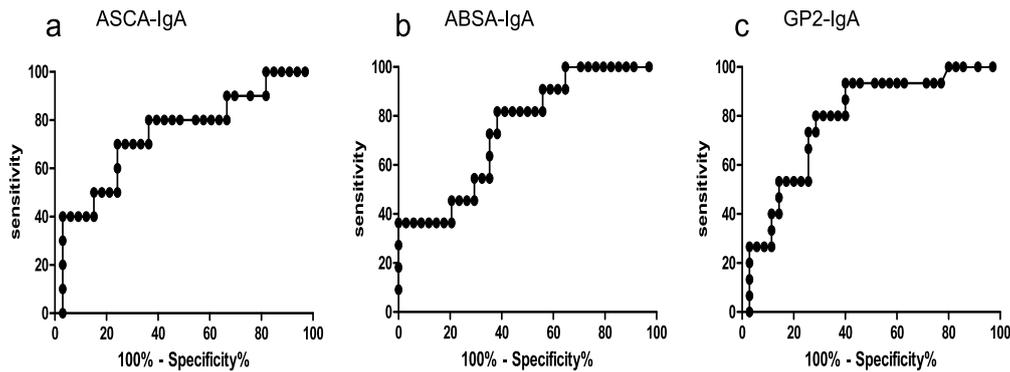


Fig. 5. Receiver Operator Curves (ROC) of a) ASCA-IgA, b) ABSA-IgA and c) GP2-IgA

Comparing ABSA-IgA, ASCA-IgA and TG2A-IgA in terms of mucosal damage revealed that TG2A had the highest sensitivity/specificity for villous atrophy in CD patients (sensitivity: 68%, specificity: 84%, $p < 0.001$), followed by ASCA (sensitivity 39%, specificity 84%, $p = 0.05$). GP2A ($p > 0.05$) and ABSA ($p > 0.05$) had a sensitivity of 13% and 43% and a specificity of 80% and 64%, respectively (Fig. 4). To explore whether the three markers were suitable to distinguish RCD patients from patients on a GFD, ROC analyses were conducted (Fig. 5). These analyses revealed that all three markers individually could discriminate RCD with AUCs between 0.74 and 0.78. Although the AUC values are not significantly different, based on the likelihood ratio's and predicted post-test probabilities within the tested population, GP2A, followed by ASCA, appears to be the superior marker (Table III). Combining these parameters did not improve the AUCs (Table III).

DISCUSSION

The objective of this study was to investigate whether GP2 antibodies can serve as biomarkers for RCD, and how GP2A relate to ABSA and ASCA in this respect. In addition we investigated whether GP2A-IgA are related to small bowel disease.

We observed elevated levels of GP2A-IgA in CrD (prevalence of 13%), active CD (prevalence of 29%) but not in UC patients (none positive), which is largely in line with previous studies describing a prevalence of GP2A-IgA-positive

patients ranging from 4.9 to 24.0% in CrD and from 2.0 to 13.0% in UC and of 38% in active CD [4-6, 9]. The prevalence of G2PA-IgG in our CrD (18.4%) and UC (12.5%) group was also in line with previous findings, i.e. 9.8 to 27.1% in CrD patients and 6.8 to 22.0% in UC patients [4-6, 9]. Interestingly, we did not observe any GP2A-IgA positive UC, and positivity for both IgA and IgG only occurred in CrD, confirming that GP2A-IgA and -IgG may be used together to differentiate CrD patients from UC. However, these markers are limited due to their low sensitivity ($< 20\%$). These data are also suggestive for an association of GP2A-IgA with small intestinal damage. However, analysis of GP2A-IgA levels within CrD subgroups with exclusive colon or ileum localized disease did not substantiate this suggestion. This is in contrast with previous findings [8, 17, 18] and may be related to the relatively small group sizes in our study.

To the best of our knowledge this is the first study which measured GP2A in CD patients on a GFD and in RCD patients. Our analyses showed that GFD patients had lower levels of GP2A-IgA than active CD patients, while RCD patients had elevated GP2A-IgA levels compared to healthy controls but more importantly compared to patients on a GFD.

As GP2 occurs in the pancreatic fluid, which finally reaches the intestines, antibodies against GP2 may be formed due to enhanced diffusion of GP2 into the lamina propria. Therefore, GP2A antibodies could be a marker for loss of intestinal integrity. In line with this theory we observed decreased GP2A-IgA levels in GFD patients. Previously Granito and

Table III. Usefulness of GP2A, ASCA and ABSA for identifying refractory CD patients from GFD patients

	CD on a GFD	Refractory CD type II	AUC	P_{AUC}	Cut off	Specificity	Sensitivity	Likelihood ratio	Predicted post-test probability
ASCA-IgA levels (AU/ml)	11.9 [5.7-38.5]	21.5 [7.8-207]	0.74 [0.55-0.93]	0.02	25 U/ml	85%	40%	2.64	44%
ABSA-IgA levels (AU/ml)	6.3 [0.4-327]	30.7 [4.4-1781]	0.75 [0.59-0.91]	0.02	28 AU/ml	71%	45%	1.55	33%
GP2A-IgA levels (AU/ml)	3.4 [0.5-30.5]	13.3 [1.3-162]	0.78 [0.64-0.91]	0.002	20 U/ml	89%	33.3%	2.92	56%
Markers positive (excluding TG2A) (n)	0 [0-3]	1 [0-3]	0.71 [0.52-0.91]	0.052					
Markers positive (including TG2A) (n)	1 [0-3]	1 [0-3]	0.64 [0.44-0.84]	0.19					

GFD = Gluten-free diet; AUC = area under the curve; P_{AUC} = p-value for AUC > 0.5 ; data shown as median [P5-P95]; TG2A= antibodies against transglutaminase-2

colleagues described an association of actin IgA antibodies and the severity on intestinal damage in active CD [19]. Similar to GP2A-IgA, anti-actin IgA antibodies disappeared after gluten withdrawal. Whether anti-actin IgA increases again in RCD remains to be investigated. Similarly to GP2A, ASCA have been investigated as markers to distinguish CrD from UC and are also found in CD [20-22]. Previously we reported that ABSA and ASCA, which are directed against luminal food antigens, decrease on a GFD as well [10], which further supports the hypothesis of antigens of luminal content being accountable for the formation of specific antibodies. Here we show an association of ASCA with villous atrophy while GP2A and ABSA did not show such an association. This may be related to the size of the antigen; *Saccharomyces cerevisiae* is larger than BSA and GP2 and may thus only reach the lamina propria when there is a higher permeability due to increased intestinal damage, while the smaller BSA and GP2 may also cross recovering epithelium. The decrease in GP2A-IgA (and ABSA-IgA) titres in patients following a GFD may not primarily depend on the recovery of the intestinal epithelial barrier, but on other factors, such as the inflammatory state of the small intestine, which is reduced upon a GFD as exemplified by a reduction in the number of intraepithelial lymphocytes. Alternatively, GP2A-IgA levels could be influenced by pathologically enhanced transcytosis of intestinal M-cells. As GP2 is expressed on human M-cells, facilitating the transcytosis of FimH(+) bacteria [23], autoantibodies against GP2 could be a result of erroneous activation of GP2-specific B-cells by bacteria-specific Th cells, similar to the erroneous activation of TG2A-specific B-cells by gluten-specific T-cells, which is characteristic for CD. Evidence for a pathologically enhanced transcytosis exists for TG2A in CD [24], and may indeed also be the case for intestinal M-cells.

Although the overall sensitivity of GP2A-IgA for RCD is relatively low (33%), increase of the titer during follow-up of a GFD may aid in the diagnosis of RCD, or indeed in the follow-up of RCD. Particularly patients with adult onset CD are at risk of developing RCD, thus in this group of patients GP2A-IgA (and ASCA/ABSA) analysis may aid in the diagnosis of RCD, especially when symptoms recur or persist despite a strict GFD (and thus a negative TG2A). In uncomplicated CD patients diagnosed at a young age, TG2A is the superior marker for follow-up of GFD and to predict mucosal recovery, GP2A-IgA analysis is not of use in this group due to the low sensitivity. Apart from the fact that the RCD patient group was relatively small, our study is particularly limited by the lack of follow-up data of patients on a GFD who developed RCD. All RCD patients included in the study were referred to our hospital when RCD already had developed.

CONCLUSION

We showed that although GP2A-IgA levels do not allow conclusions on the histological status of the intestinal mucosa, GP2A-IgA as well as ASCA-IgA and ABSA-IgA levels are enhanced in refractory CD patients as compared to patients on a GFD. Analysis of these antibodies in the follow-up of CD patients may be useful to detect patients at risk to develop refractory CD.

Conflicts of interest. None to declare.

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