

Antibodies against laminaribioside and chitobioside are novel serological markers in Crohn's disease

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Abbreviations: ACCA, anti-chitobioside carbohydrate IgA antibodies; ALCA, anti-laminaribioside carbohydrate IgG antibodies; 5-ASA, 5-aminosalicylic acid; ASCA, anti-*Sacharomyces cerevisiae* antibodies; AUC; area under the curve; CD, Crohn's disease; gASCA; anti-covalently attached mannan antibodies; HC, healthy controls; IUPAC, International Union of Pure and Applied Chemistry; 6-MP, 6-mercaptopurine; MTX, methotrexate; OD, optical density; OGD, other-than-IBD gastrointestinal diseases; RFU, relative fluorescence units; SD, standard deviation; UC, ulcerative colitis;

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Abstract

Background and aims: New serological markers of inflammatory bowel disease may be useful for differentiating between Crohn's disease and ulcerative colitis, and for disease stratification. We profiled sugar-binding antibodies in order to identify novel anti-glycan antibodies that may be associated with inflammatory bowel disease.

Methods: Serum samples were obtained from patients with diagnosed Crohn's disease or ulcerative colitis and from control patients. The presence of anti-glycan antibodies was evaluated using either a glycan array (GlycoChip[®]) in patients with Crohn's disease (n=72) or ulcerative colitis (n=56) and in healthy controls (n=41) or using an enzyme-linked immunosorbent assay in patients with Crohn's disease (n=124), ulcerative colitis (n=106) and in control patients (n=101).

Results: In addition to antibodies against mannan, antibodies to laminaribioside (Glc(β1,3)Glc(β)) and chitobioside (GlcNAc(β1,4)GlcNAc(β)) had the highest discriminative capability between Crohn's disease and ulcerative colitis ($p < 0.001$ and < 0.05 , respectively). Importantly, 44% (12/27) of anti-*Sacharomyces cerevisiae* antibody negative Crohn's disease patients were positive for anti-laminaribioside or anti-chitobioside. In patients with inflammatory bowel disease positive for antibodies against either laminaribioside, chitobioside or mannan, the diagnosis of Crohn's disease was suggested with a sensitivity of 77.4% and specificity of 90.6%. Having at least two of these antibodies increased the specificity to 99.1%. In Crohn's disease, higher levels of antibodies against laminaribioside or mannan were significantly associated with small intestinal disease ($p = 0.03$ and $p < 0.0001$, respectively).

Conclusions: Anti-laminaribioside and anti-chitobioside carbohydrate antibodies are novel serological markers associated with Crohn's disease. These antibodies may contribute to the diagnosis and improved stratification of Crohn's disease.

Introduction

Inflammatory bowel disease (IBD) is a chronic intestinal disorder of unknown etiology comprising two major types: Crohn's disease (CD) and ulcerative colitis (UC). The diagnosis of IBD and the differentiation between UC and CD are established by the combination of clinical, laboratory, radiological, endoscopic, histopathologic and serological characteristics. However, when a definite diagnosis cannot be established, as is true in 10–17 % of colitis patients, a diagnosis of indeterminate colitis (IC) is designated.¹⁻⁵

In addition to diagnosing IBD, serological markers may be used to discriminate between CD and UC, assess disease activity and progression, predict response to treatment, and to stratify disease severity.⁶ The major serological markers for IBD in commercial use are anti-neutrophil cytoplasmic antibodies (ANCA) and anti-*Sacharomyces cerevisiae* antibodies (ASCA). ANCA are autoantibodies directed at antigens found mostly in azurophilic granules of neutrophils,^{7,8} and have a prevalence of up to 85% among UC patients and 5–28% among CD patients and healthy people. ASCA are directed against mannose residues on mannan in the cell walls of the yeast *S. cerevisiae*^{9,10} and have a prevalence of 48–69% among CD patients and 15% among UC patients.^{7,10}

It has been demonstrated that serological markers are associated with phenotypic subsets of CD. ASCA is associated with severe small-bowel disease rather than colonic disease and with penetrating CD, whereas ANCA-positive CD patients have a more “UC-like” clinical manifestation.¹¹⁻¹³ Other antibody markers have been reported in serum samples from IBD patients, including those against outer-membrane porin C (OmpC) of *E. coli*, found in 38-55% of CD and 2% of UC patients, against the bacterial sequence I2, found in 54% of CD and 10% of UC patients, and against bacterial flagellin (CBir), in CD, in contrast to UC patients.¹⁴⁻¹⁷ These antibodies, directed at bacterial

antigens, are also associated with distinct disease phenotypes, specifically more complicated small bowel disease.^{17, 18} However, the significance of serological markers for discrimination between UC and CD, and their significance in better classifying IC, remains controversial.^{2, 19, 20} Therefore, there remains a considerable unmet need for serological markers in IBD.

The existence of antibodies against sugars, such as ASCA, that are CD-specific, and the need for better serological stratification of IBD prompted us to perform an extensive anti-glycan antibody search in IBD patients. Glycans are predominant surface components of cells such as erythrocytes, immune cells, and microorganisms. As such, they give rise to high levels of anti-glycan antibodies of all classes, which have been demonstrated in a number of inflammatory and autoimmune diseases.²¹⁻²³

To investigate anti-glycan antibodies as potential serological markers in IBD, we first used an array of sugars (mono-, di-, and trisaccharides) to characterize the anti-glycan antibody profile in serum samples from CD and UC patients and from healthy controls (the discovery phase). We then screened serum samples from IBD patients and controls to detect the presence of the characteristic anti-glycan antibodies (the validation phase). Here, we report on the discovery of two novel anti-glycan antibodies, laminaribioside and chitobioside, that appear to be associated with CD.

Materials and methods

Patients

Patients (N=331) were recruited at the Department of Gastroenterology and Liver Diseases, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel. Signed informed consent forms were obtained from all patients and the local ethical committee approved the study. The diagnosis of UC or CD was based on established clinical, radiographic, endoscopic, and histopathologic criteria. Patient characteristics were determined from medical records, questionnaires and interviews. Ashkenazi or Sephardic origins were assigned on the basis of the birthplace of the four grandparents. Mixed origin is the case for which at least one grandparent belonged to a different ethnic Jewish group than the other grandparents. Patients were considered current or past smokers if they smoked a minimum of seven cigarettes per week for at least one year anytime during their life. CD patients were assigned phenotypes based on previously published criteria.²⁴ Disease behavior was divided based on the Vienna classification into non-stricturing non-penetrating (inflammatory), stricturing, and penetrating disease types. Disease location was defined as the presence of inflammation in the small bowel, colon or both on the basis of endoscopic, radiological or histopathological studies. Phenotypes and disease locations were assigned by IBD physicians once the disease was already established. The control groups consisted of healthy blood donors (HC), and, for the validation screening (see in "serological analysis") we also added a non-IBD gastrointestinal diseases control group (OGD, n=61). This group consisted of celiac disease patients (n=27) confirmed by serology (antibodies to tissue transglutaminase and to endomysium) as well as by duodenal biopsies, irritable bowel syndrome (IBS) patients (n=20), and 14 patients with colonic polyps, diverticular disease, pseudomembranous colitis, *Helicobacter pylori* gastritis, pancreatitis, and hemorrhoids; Patient characteristics are presented in Tables 1 and 2.

Serological analysis

Blood samples were collected in evacuated silicon-coated tubes containing gel for easier separation between serum and blood clot (Cat. 418XX3GLV, Estar Technologies, Holon, Israel). After blood coagulation, serum was separated by centrifugation at 100g, collected, and kept frozen at -80°C until use. The laboratory evaluations were conducted in a blinded manner.

Discovery phase: preliminary screening

Determination of anti-glycan antibodies profile using a glycan array

Profiling of anti-glycan antibodies in serum samples was done using GlycoChip[®], a glycan array in the 384-well microtiter plate format, as previously described.²⁵ Briefly, p-nitrophenyl saccharides were covalently bound to the surface of the microtiter plate wells (384-well reduced-volume black microtiter plate; Greiner Bio-One international AG, Kremsmuenster, Austria) with a linker (an oligomer of 1,8-diamino-3,6-dioxaoctan (DD8); Sigma, St. Louis, MO, USA). The mono-, di-, oligo-, and polysaccharides used are listed in Table 3.

The volume of all solutions added to the glycan array was 10 μL /well, except where stated. Serum samples were diluted 1:40 in 0.15 M Tris-HCl, pH 7.2, 0.085 M Mg_2SO_4 , 0.05% Tween-20 (TBST) containing 1% bovine serum albumin (BSA; Sigma), dispensed into the plates using a Tecan Genesis Workstation 200 automated handling system (Tecan, Zurich, Switzerland), and incubated for 60 min at 37°C . The plates were then washed with 250 μL /well phosphate-buffered saline (PBS) with 0.05% Tween-20 (PBST; Sigma) in an automatic plate washer (PowerWasher[™], Tecan, Zurich, Switzerland). The respective isotype-specific biotinylated goat anti-human Ig antibodies against IgG, IgA or IgM (Jackson ImmunoResearch Laboratories, West Grove, PA, USA)

were then added, and incubated for 60 min at 37°C. Following washing with PBST, streptavidin-conjugated europium 0.1 µg/mL (Perkin-Elmer, Boston, MA USA) was added for 30 min incubation at 37°C in the dark and washing with PBST. Delfia™ enhancement solution (Perkin-Elmer) was then added and plates were incubated 30–45 min in the dark at room temperature. Fluorescence was read with a Victor™ 1420 multi-label counter (Perkin-Elmer) using time-resolved fluorescence settings of 340/612 nm (excitation/emission).

In order to determine positive reactivities for the results obtained from the glycan array we used relative fluorescence units (RFU). The cut-off values were 2.5×10^6 RFU for IgG and 0.74×10^6 RFU for IgA. These cut-off values were determined using receiver operating characteristic (ROC) curves (data not shown) to provide optimal positive and negative predictive values for differentiation between CD and UC patients for the relevant antibodies.

Validation screening

Determination of anti-glycan antibodies using an ELISA-based assay.

After profiling anti-glycan antibodies by the glycan array using indirect immunofluorescence, we established an ELISA assay for the determination of the three most discriminative antibodies, thus facilitating wide-scale screening. Briefly, mannan and p-nitrophenyl derivatives of chitobioside and laminaribioside (the three glycans towards which most prominent and discriminative seroreactivities were detected in the discovery phase) were covalently attached to the surface of a 96-well microtiter plate via a linker as previously described.²⁵ Serum samples were diluted 1:400 in 5% DD8/1%BSA/TBST, pH 7.4, dispensed into the wells (50 µL per well) incubated for 30 min at 25°C, then washed with PBST buffer. Bound antibodies were labeled (30 min at 25°C) with 50 µL

of either horseradish peroxidase (HRP)-conjugated goat anti-human IgA (1:2500) or IgG (1:5000) type-specific antibody (both antibodies from Jackson, ImmunoResearch Laboratories, West Grove, PA, USA), washed with PBST buffer, and 50 μ L 3,3',5,5'-tetramethylbenzidine (TMB) was added for detection. The optical density (OD) at 595 nm was read after 6 min with a Victor 1420 plate reader (Wallac, Turku, Finland), the enzymatic reaction was stopped with 50 μ L TMB 'stop' solution and read at 450 nm. Serum samples were considered as positive for IgA anti-chitobioside (ACCA), IgG anti laminaribioside (ALCA), and IgG anti-covalently attached mannan (gASCA) if OD values at 450 nm were above the following cut-off values: 0.43, 1.55, and 0.4 respectively. These cut-off values were determined using ROC curves (Figure 4) to provide 97%, 100%, and 92% specificity for ACCA, ALCA, and gASCA, respectively, for differentiation of CD and UC patients.

Determination of ASCA

Serum levels of ASCA were determined by ELISA using the QUANTA Lite™ ASCA IgG kit according to the manufacturer's protocol (Inova Diagnostics Inc., San Diego, CA, USA). The results were presented as arbitrary ELISA units (EU) that were calculated by dividing the average optical density of the sample by the average optical density of the ASCA IgG ELISA low positive. The result was multiplied by the number of units assigned to the ASCA IgG ELISA low positive and the results were expressed as negative (<25 EU), or positive (>25 EU).

Statistical analysis

Descriptive statistics were used for population and subgroup characteristics. For results obtained using the glycan array, ROC curves (generated by plotting sensitivity vs. (1-

specificity)) were calculated for the GlycoChip® data. The curves were used to determine cut-off values providing optimal positive and negative predictive values.

For comparison between the study groups, and testing association with clinical parameters, OD results were transformed using log10 for achieving distributions as close as possible to normal distribution. We have used Student's T-test to assess significance differences in anti glycan antibodies between groups, and χ^2 test for non-parametric variables. Pearson correlation was used to test association between antibody levels and age or gender; a p -value < 0.05 was considered to be statistically significant.

To evaluate the association between disease phenotypes and the level of immune response toward the three glycan antigens- laminaribioside, chitobioside, and oligomannan simultaneously, we performed quartile sum analysis¹⁷. For each antigen, patients whose antibody levels were in the 1st, 2nd, 3rd, and 4th quartile of the distribution were assigned a quartile score of 1, 2, 3, and 4, respectively. By adding individual quartile scores for each glycan antigen, a quartile sum score (range, 3–12) was obtained to represent the cumulative quantitative immune response toward all 3 antigens for each patient¹⁸. The Cochran–Armitage test for trend was used to test whether there was a linear trend in the proportion of patients with a disease phenotype characteristic as the level of antibody responses increased by quartiles. A P value (P trend) < 0.05 suggests that the linear trend is statistically significant.

Results

Discovery phase: preliminary screening

Patient characteristics

Serum samples were obtained from patients with CD (n=72) and UC (n=56), and healthy controls (n=41). There were no significant differences in demographic parameters between the groups studied (Table 1). As expected, bowel operations and the use of biological treatments (Infliximab) were significantly more frequent in the CD than the UC group ($p<0.0001$ and $p=0.049$, respectively), whereas the use of 5-aminosalicylic acid (5-ASA) was significantly higher in the UC group ($p=0.0001$).

Anti-glycan antibodies in IBD and healthy control patients.

Serum samples were initially screened for 48 different glycans using the GlycoChip® glycan array (Table 3). In addition to antibodies against mannan and mannan residues, one IgG and one IgA anti-glycan antibodies were significantly elevated in the sera of CD patients compared with UC patients ($p<0.001$) (Tables 4 and 5). Anti-Glc(β1,3)Glc(β) (laminaribioside) IgG and anti-GlcNAc(β1,4)GlcNAc(β) (chitobioside) IgA were the most prominent antibodies, demonstrating the highest mean relative fluorescence units (RFU) in the CD group, and the most significant p -values for discrimination between CD and UC patients. Anti-Glc(β1,3)Glc(β) (laminaribioside) IgG was also significantly higher in CD patients compared with healthy controls ($p=0.05$), while anti-GlcNAc(β1,4)GlcNAc(β) (chitobioside) IgA was comparable between CD patients and healthy controls ($p=0.15$). These novel antibodies were thus designated anti-laminaribioside carbohydrate IgG antibodies (ALCA) and anti-chitobioside carbohydrate IgA antibodies (ACCA). The distribution of ALCA and ACCA in CD, UC, and healthy control patients is described in Figure 1. The binding specificities of ALCA and ACCA were verified by inhibition studies using the relevant soluble antigens (data not shown).

The percentage of the CD cohort that was positive for ALCA, ACCA, and ASCA, was 37.5, 36, and 65, respectively. A total of 20.8% of the CD patient cohort were seronegative for all three anti-glycan antibodies.

The ASCA results were compared with binding levels of antibodies against mannan residues on the GlycoChip[®] glycan array. The two assays had a correlation of $R^2=0.76$, further validating the GlycoChip[®] assay.

No anti-glycan antibodies were elevated in UC patients compared with CD patients or healthy controls. There were no significantly elevated anti-glycan IgM antibodies in the groups studied (data not shown).

Lack of correlation between anti-laminaribioside or anti-chitobioside carbohydrate antibodies (ALCA and ACCA) and anti-mannan antibodies

Different seroreactivities to various glycans may suggest not only different epitopes but also the possibility of different patient subpopulations. Thus, the correlation between seroreactivities to mannan, laminaribioside, and chitobioside within the CD patient cohort was assessed. There were poor correlations between levels of anti-mannan IgG antibodies and ALCA ($R^2=0.26$), between levels of anti-mannan IgG antibodies and ACCA ($R^2=0.18$), and between levels of ALCA and ACCA ($R^2=0.03$). The low correlations suggest minimal serological overlap. Thus, anti-glycan antibodies may define different CD serotypes.

ALCA and ACCA reactivities in ASCA-negative CD patients

It was previously suggested that ASCA-negative CD patients may represent a distinct phenotypic group compared with ASCA positive patients.¹² Importantly, 12/27 (44%) of

CD patients who were ASCA-negative were ALCA- or ACCA-positive (Figure 2). Thus, the combined use of the three antibodies ALCA, ACCA, and ASCA may increase the sensitivity of diagnosing CD.

Validation screening

Patients

Using the same inclusion criteria as described for the discovery screening, we extended the study population to 124 CD and 106 UC patients, and included a control group of other non-IBD gastrointestinal diseases (OGD; n=61) including 27 celiac disease patients, 20 patients with irritable bowel syndrome (IBS) and 14 patients with colonic polyps, diverticular disease, pseudomembranous colitis, *Helicobacter pylori* gastritis, pancreatitis, and hemorrhoids, as well as apparently healthy blood donors (HC; n=40). The characteristics of these groups are summarized in Table 2.

ALCA, ACCA, and gASCA levels in CD, UC, and control patients

Serum samples were screened using an ELISA-based assay to determine levels of ACCA, ALCA, and IgG antibodies against covalently immobilized mannan (gASCA). ALCA levels were significantly higher in CD compared with all other groups: $p < 0.0001$ vs. UC and healthy controls, and $p < 0.05$ vs. non-IBD gastrointestinal diseases (OGD) (Figure 3a). ACCA levels were significantly higher in CD than in UC ($p < 0.0001$) and OGD groups, but comparable to the apparently healthy blood donor group (Figure 3b). ACCA levels in UC were significantly lower also when compared to the healthy blood donors group ($p = 0.004$). Levels of gASCA were significantly increased in CD patients compared with all other groups ($p < 0.0001$; Figure 3c).

ALCA, ACCA, and gASCA were positive in 27, 25, and 69 percent of the CD cohort, in 4, 5, and 7 percent of the UC cohort, in 9, 9 and 13 percent of the OGD

cohort and in 2, 12 and 15 percent of healthy control patients, respectively (see table 7 for cut-off values). Of note, it was previously reported that ASCA may be present in celiac disease patients^{26,27}. It is intriguing that in the subgroup of 27 celiac disease patients who were included in the OGD control group, gASCA, ACCA and ALCA were positive in 6/27 (22%), 6/27 (22%) and 2/27 (7%), respectively. More importantly, only one celiac disease patient was double positive (gASCA and ALCA) and there were no celiac patients who were triple positive for anti-glycan antibodies.

The sensitivity, specificity, positive and negative predictive values and efficiency for differentiation between CD and UC, and between CD and control patients (OGD and healthy controls) based on the combination of ALCA, ACCA, and gASCA are described in Tables 6 and 7, respectively. In comparison to the use of commercial ASCA, the combined use of ALCA, ACCA, and gASCA improved the ability to differentiate CD from UC patients; CD patients who were positive for at least one of the antibodies could be differentiated from UC patients with a sensitivity of 77.4%, and a specificity of 90.6%. Moreover, the 26.4% of the CD patients who were positive for two or more anti-glycan antibodies had a specificity of >99% for the diagnosis of CD.

Receiver operating characteristics (ROC) curves were generated by plotting sensitivity vs. (1-specificity), thus allowing optimisation of decisions regarding sensitivity and specificity for each of the tests performed. ROC curves comparing discrimination between CD and UC patients and between CD and control patients (OGD and healthy controls) based on levels of ALCA, ACCA and gASCA are shown in Figures 4 and 5, respectively.

Overall, ALCA gave better discrimination between CD and UC (area under the curve [AUC]=0.813) compared with ACCA (AUC=0.689), as shown in Figures 4-5.

Association of ALCA, ACCA, and gASCA to disease phenotype in CD

An association between CD phenotypes and various seroreactivities was previously reported,^{11, 18} as well as an association of higher antibody levels with distinct disease behaviors, such as complicated small-bowel disease and the risk for early surgery.^{18, 28} We used the Vienna classification²⁴ in order to analyze a possible association between the seromarkers and disease behavior. In the non-stricturing non-penetrating (inflammatory) group, out of 57 patients, ALCA, ACCA and gASCA positivities were 25, 18 and 61 percent, respectively. In the stricturing group, out of 38 patients 26, 34, and 76 percent, respectively, were positive and in the penetrating group, out of 29 patients 34, 29, and 76 percent, respectively were positive.

In our CD patient cohort, higher levels of ALCA and gASCA were significantly associated with disease localization to the small intestine compared with colonic disease ($p < 0.05$ and $p < 0.0001$, respectively), and with the more severe, penetrating or stricturing compared with non-stricturing non-penetrating disease phenotype ($p = 0.04$ and $p = 0.008$, respectively), whereas ACCA levels had no significant association for disease localization or behavior (Tables 8 and 9). Furthermore, as seen in Table 10, patients with high antibody levels against multiple glycan antigens (increasing quartile sum scores) tended to have an increased likelihood of small bowel disease (p trend = 0.001) as well as penetrating or stricturing phenotype (p trend = 0.054). Not only were high anti-glycan antibody levels, but also the mere presence of an anti-glycan antibody was associated with specific disease localization. Of the patients who were negative for all three seromarkers, 17/28 (60%) had small bowel disease, whereas 48/51 (94%) who had at least one positive, and 42/45 (93%) who had 2-3 positive seromarkers had small bowel disease (p trend < 0.001, odds ratio 2-3 positive antibodies vs. 0 positive is 9.0, CI 95% 3.3-24.5).

The increase in ALCA and gASCA levels in the more severe disease phenotypes (stricturing or penetrating group) vs. non stricturing, non penetrating group supports previous observations²⁹ where an increase in ASCA positivity and titers¹¹ in the more aggressive disease phenotypes was noted.

There was no correlation between any of the three antibodies with CDAI, treatment regimen, smoking status, origin, family history of IBD, and no phenotypical difference between the gASCA negative and gASCA positive patients subgroups was noted.

Discussion

We have described two novel anti-glycan antibodies, specifically associated with CD, which were discovered using a GlycoChip[®] glycan array. This technique uses a structurally defined set of mono-, oligo- and polysaccharides linked to an inert surface, mimicking the presentation of glycosylated molecules on living cells. One antibody that is significantly increased in CD, compared with UC patients and healthy controls, is an IgG antibody directed against the glucose-based glycan laminaribioside – designated anti- laminaribioside carbohydrate antibody (ALCA). The other is an IgA antibody, directed against the *N*-acetyl glucosamine-based glycan chitobioside – designated anti-chitobioside carbohydrate antibody (ACCA). In addition to ALCA and ACCA, antibodies to mannan and mannan residues were significantly increased in CD compared with UC patients, as expected (given the known association with ASCA), thus further validating our screening.

A significant subgroup of CD patients has negative seroreactivity to mannans, reflected in the low sensitivity of ASCA in CD patients. Most reports suggest sensitivities of approximately 60%; however, values as low as 39% and 44% have also been reported.^{7,30,31} Thus, additional seromarkers are needed to improve the diagnosis and stratification of CD. Indeed, it was recently reported that certain antibodies against bacterial components may be used as serological markers for the diagnosis of CD. Antibodies against OmpC of *E. coli*, against the Crohn's disease-related bacterial sequence I2 and against bacterial flaggellin were detected in 38-55%, 50% and 52% of CD patients, respectively^{14,15,17,32} thus further increasing the diagnostic accuracy of Crohn's disease, especially in ASCA-negative cases.¹⁸ In our discovery cohort, 37.5% of the CD patients tested were positive for ALCA and 36% for ACCA. Importantly, 44% of the ASCA-negative CD patients in our study were ALCA- or ACCA-positive. Even in

the validation cohort, where higher cut-off values were used in order to increase specificity, there were 10/38 (26%) gASCA-negative ALCA or ACCA positive patients, suggesting that the combination of ALCA, ACCA, and gASCA may improve the serological diagnosis of CD. The small number of patients in the gASCA-negative, ALCA or ACCA positive group may account for the lack of statistically significant differences in phenotype between these groups.

Laminaribioside is the building block of laminarin, a polysaccharide of the β -1-3-glucan family. Beta-1-3 glucans may be found in the cell walls of saprophytic and pathogenic fungi and yeast, including *S. cerevisiae*, as well as in food (oats) and algae.³³ Chitobioside is a component of chitin, a polymer of *N*-acetyl- β -D-glucosamine, a major component of the insect cuticle as well as the cell walls of infectious pathogens such as bacteria and yeast.³⁴ Both β -1-3 glucans and chitin may bind to specific receptors on neutrophils, macrophages and NK cells, thereby stimulating cell proliferation, phagocytosis and cytokine secretion.^{35,36} β -1-3 glucan was reported as the only ligand for Dectin-1, a lectin-like receptor on leukocytes, while there was no Dectin-1 binding for another 187 predominantly mammalian-type glycans. Dectin-1 binding was associated with TNF- α secretion by a macrophage cell line as well as T lymphocyte proliferation³⁷. Chitin is the substrate for chitotriosidase (chitinase 1), an enzyme in macrophages and neutrophils that is regulated by GM-CSF and its chitin degrading activity increases in systemic fungal infections³⁸, as well as in multiple sclerosis³⁹. Even more intriguing is the recent study by Mizoguchi⁴⁰ reporting on the upregulation of chitinase 3-like-1 (CHI3L1), a member of the mammalian chitinase family that lacks catalytic activity, but has preserved binding ability to chitin. This protein is expressed by lamina propria lymphocytes as well as intestinal epithelial cells, and is upregulated in murine and human intestinal inflammation i.e. DSS colitis, CD and UC. This enzyme serves as an enhancer,

rather than an inhibitor, of bacterial adhesion and invasion. Thus, β -1-3 glucans and chitin are glycans that have the distinct potential to modulate the immune system, specifically its innate arm while the finding of antibodies against these components that are specifically associated with CD suggests a link to adaptive immunity.

A CD associated immune response against microorganism derived antigens such as laminaribioside and chitobioside is consistent with the reports on ASCA^{9,10}, OmpC¹⁴, I2¹⁵, and flaggellin.^{16,32} Whether these antibodies target antigens that have a role in the immunopathogenesis of IBD or are they merely markers of the inflamed leaky bowel in CD is a fundamental question. Data from several directions suggest that loss of tolerance, rather than an increase in intestinal permeability is the major mechanism; Lack of correlation between serologic CD phenotypes and intestinal permeability was reported by Vermeire *et al.*⁴¹, and Harrer *et al.*⁴² suggesting that serologic markers are not an epiphenomenon related to disease activity. Lindberg *et al.*⁴³ demonstrated that CD was associated with a differential antibody response that was high towards yeast and mannan but normal towards food antigens, and Konrad *et al.*⁴⁴ had shown a specific and strong lymphocyte reactivity towards mannan compared with the food antigen ovalbumin in ASCA positive CD patients, both suggesting that the anti-microorganism response in CD is not merely due to a generalized immunologic hyperreactivity towards intestinal contents, as would be expected if intestinal permeability were the main mechanism. Israeli *et al.*⁴⁵ assessed serum samples from apparently healthy recruits to the Israeli Defence Forces, who were later diagnosed as having CD. Interestingly, they show that ASCA was positive in 90% of patients in the first serum samples i.e. before CD diagnosis. Moreover, the mean interval between ASCA detection and the diagnosis of CD was >3 years. This observation suggests that ASCA is not merely a marker of disease activity, and would also argue against the "leaky bowel" mechanism as it was detected in completely asymptomatic young people (although no bowel permeability evaluation was

performed in this study). The lack of correlation between seroreactivity to ALCA or ACCA and disease activity in our study are consistent with the above mentioned reports, and suggest that these antibodies, targeting glycans on microorganisms, are not markers of disease activity, but rather, may have a role in its immunopathogenesis.

The low correlation between the presence of antibodies against mannan, laminaribioside, and chitobioside suggests that the antigens responsible for these anti-glycan antibodies are not on the same organism, such as a single bacterium, whereas a high correlation between these anti-glycan antibodies would be expected if a single source were responsible. This low correlation also suggests that there are serologically defined sub-groups of CD patients. This is further supported by the demonstration that only a minority of patients (4-8%) reacts to all three anti-glycan antibodies. Similarly, Landers *et al.* showed that 26% of CD patients expressed antibodies to ASCA, OmpC and I2.¹⁷ Thus, ALCA and ACCA may also contribute to disease stratification into more homogeneous subgroups. In this respect, the sub-group of gASCA-negative, ALCA or ACCA positive patients could be of particular interest. In our cohort, however, there were no statistically significant phenotypic differences between these patients and ASCA positive patients (perhaps because of the small numbers of patients). Larger-scale studies are needed in order to clarify if this subgroup is indeed phenotypically distinct.

An alternative explanation for differences in serological reactivities is genetic heterogeneity. However, much debate remains regarding the correlation between serological markers and genetic alteration in CD, as direct association between those markers and the NOD2/CARD15 variants was not demonstrated by groups from either Europe or North America^{18,46}. Moreover, in a twin-study recently published⁴⁷ there was no correlation between ASCA and CARD15/NOD2 genotype, lending more support to the concept that anti microbial antibodies are not determined by CARD15/NOD2, and represent a response to an environmental antigen. The potential correlation between the

novel serological markers described herein and genetic heterogeneity remains to be determined.

As CD patients who were positive for at least one of ALCA, ACCA, or gASCA could be differentiated from UC patients, with 77.4% sensitivity and 90.6% specificity, and patients who were positive for two or more antibodies could be identified with specificity near 100%, anti-glycan antibodies may also be beneficial in the discrimination between CD and UC. Another group that poses a diagnostic challenge to CD is the celiac disease subgroup, where the presence of ASCA was reported to be as high as 50%^{26,27}. In our celiac patients subgroup 6/27 patients were gASCA positive, however only one was ALCA positive as well, thus contributing to the high diagnostic specificity of anti-glycan antibodies.

The association of high ALCA levels with small-bowel disease as well as the more aggressive disease phenotypes and the increased trend towards small bowel disease and an aggressive disease phenotype in patients having higher levels of the three anti-glycan antibodies (reflected in the higher quartile sum scores) may have clinical implications. This observation is similar to that reported with ASCA, OmpC, and I2, whereby anti-microbial components-positive patients with CD had more small-bowel disease and a more severe phenotype, reflected in more penetrating disease and a need for more operations.^{18,46} Thus, similarly to previously reported observations regarding the association of positive seroreactivities to microorganism components and response to treatment- such as antibiotics⁴⁸, ALCA or ACCA positivity may contribute to different disease management.

In conclusion, this study suggests that the anti-glycan antibodies ALCA and ACCA may be important serological markers for CD. Their combined use, together with that of ASCA, may improve the differentiation between CD and UC, and better stratify CD. The identification of these antibodies is consistent with the hypothesis that CD is

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associated with a defect in tolerance to commensal microorganisms. Further studies are required to explore the clinical significance of such serological markers for the diagnosis, prognosis, and disease management of IBD.

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Figure legends:

Figure 1. The distribution of anti-laminaribioside carbohydrate antibodies (ALCA) and anti-chitobioside carbohydrate antibodies (ACCA) in IBD patients and healthy controls.

Serum samples from 72 CD and 56 UC patients and 41 healthy controls were screened for the presence of anti-glycan antibodies. The two most discriminative antibodies were (a) ALCA and (b) ACCA. Box plots include signals of 50% for the relevant patient population. The median value is indicated inside the box. The lower border represents the 25th percentile, and the upper border represents the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles.

* $p < 0.05$ versus CD

** $p < 0.001$ versus CD

Figure 2. Relationship between ALCA, ACCA, and ASCA in the CD cohort by presence vs. absence.

Percentage of the CD patient cohort (n=72) that is positive for each marker, any combination of two markers, and all markers is shown. ALCA and ACCA were determined by the glycan array and ASCA by the commercial ASCA kit (Inova Diagnostics), as described in the materials and methods section. A total of 44% of ASCA-negative CD patients were positive for ALCA or ACCA.

Figure 3. The distribution of ALCA, ACCA, and gASCA in IBD patients and controls.

Serum samples from 124 CD, 106 UC and 61 non-IBD gastrointestinal disease patients (OGD) and 40 healthy controls were screened for the presence of (a) ALCA, (b) ACCA, and (c) gASCA by ELISA as described in the materials and methods section.

The median values for each group are indicated by the short horizontal lines. The crossing line indicates the cut-off value used for defining positive and negative results. OGD group included 27 celiac disease, 20 irritable bowel syndrome (IBS) patients and 14 patients with colonic polyps, diverticular disease, pseudomembranous colitis, *Helicobacter pylori* gastritis, pancreatitis, and hemorrhoids;

* $p < 0.05$ versus CD

** $p < 0.0001$ versus CD

$p = 0.004$ versus UC

Figure 4. Discrimination between CD and UC patients based on ALCA, ACCA, and gASCA levels.

Serum samples from 124 CD and 106 UC patients were screened by ELISA as described in the materials and methods section. Receiver operating characteristics (ROC) curves were generated by plotting sensitivity vs. (1- specificity), thus allowing optimization of decisions regarding sensitivity and specificity for each of the tests performed. AUC, representing the overall performance of a single marker was similar for ALCA and gASCA (0.813 and 0.849, respectively).

Figure 5. Discrimination between CD and control patients based on ALCA, ACCA, and gASCA levels.

Serum samples from 124 CD and 101 control patients (including OGD, n=61; HC, n=40) were screened by ELISA as described in materials and methods. Receiver operating characteristics (ROC) curves were generated by plotting sensitivity vs. (1-specificity), thus allowing optimization of decisions regarding sensitivity and specificity for each of the tests performed. AUC, representing the overall performance of a single marker, was 0.855 for gASCA and lower (0.720) for ALCA.

Figure 1

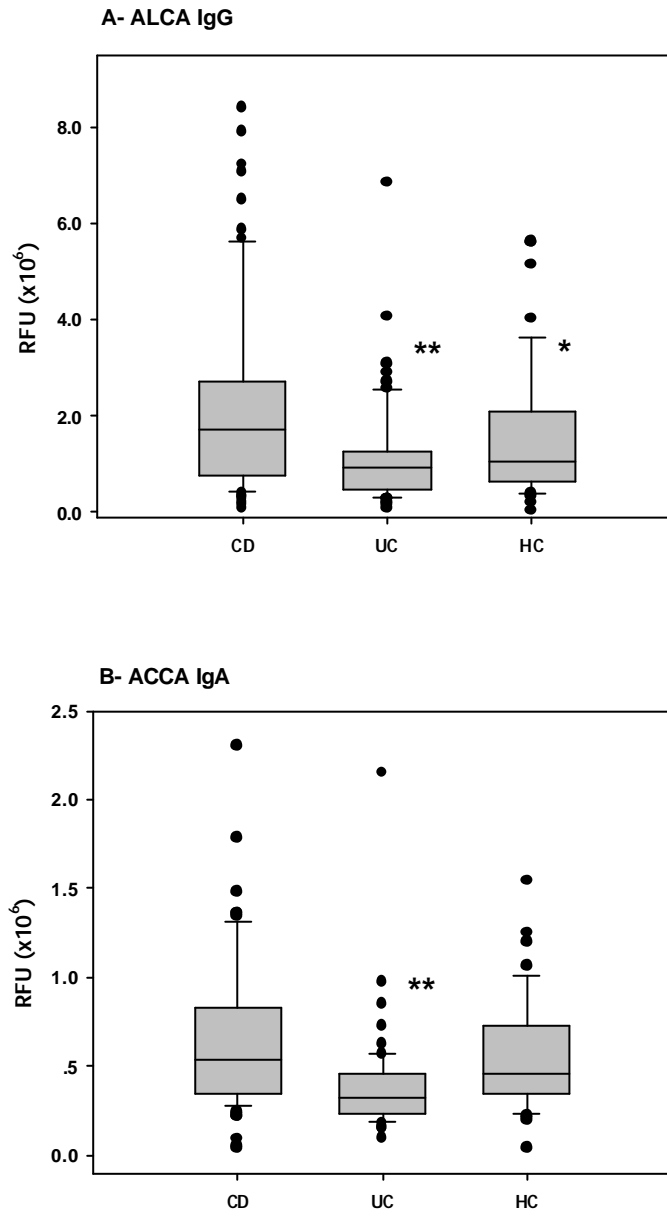


Figure 2

CD Cohort (n=72)

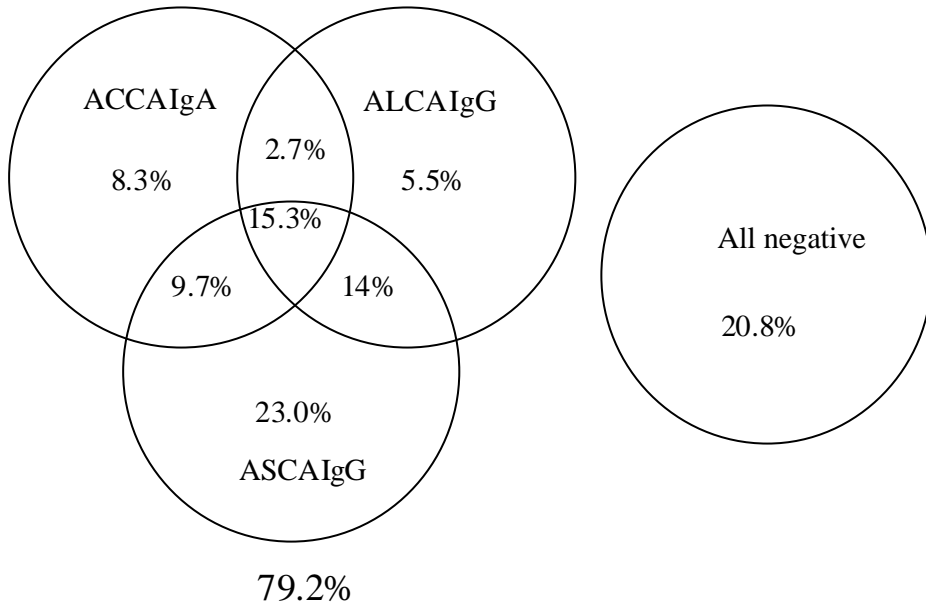


Figure 3

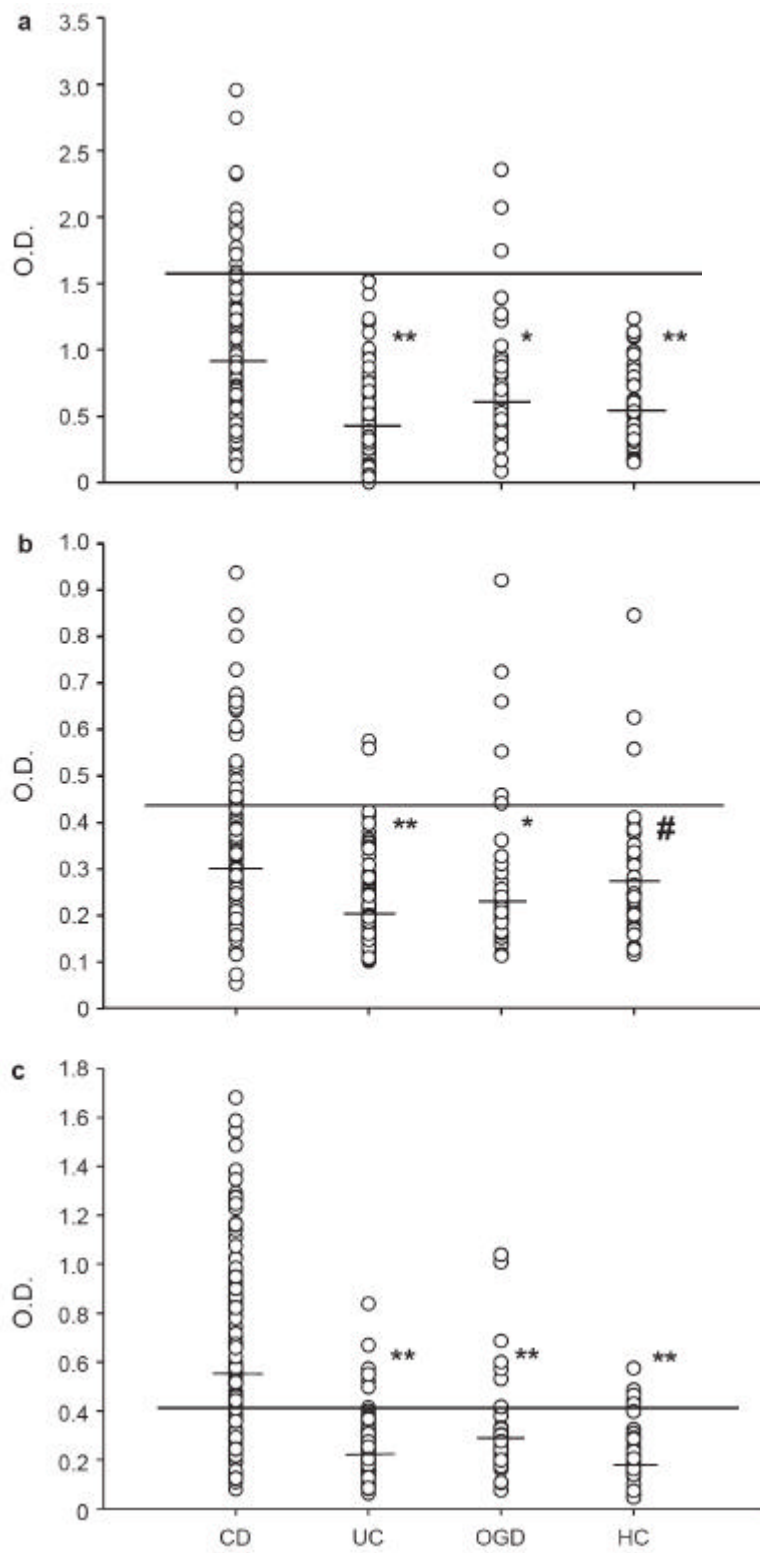


Figure 4

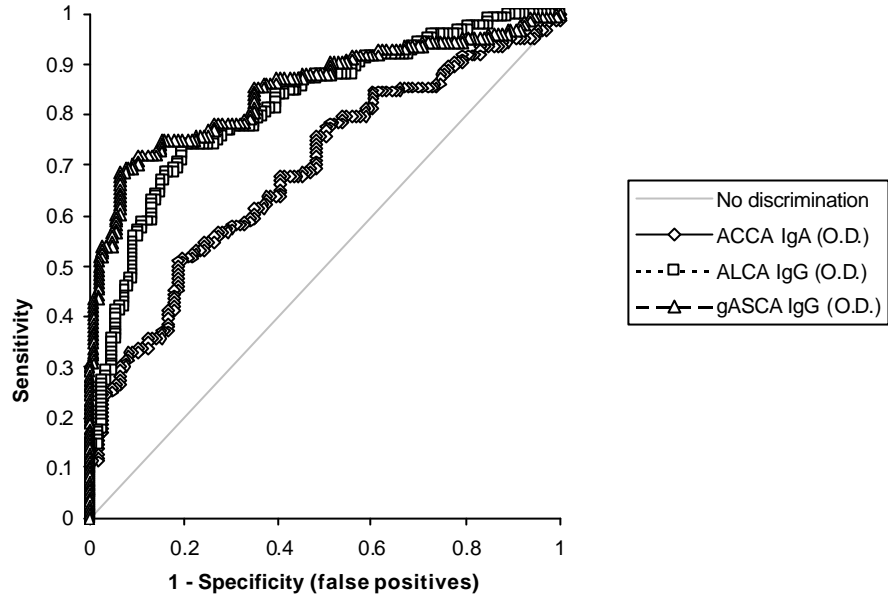


Figure 5

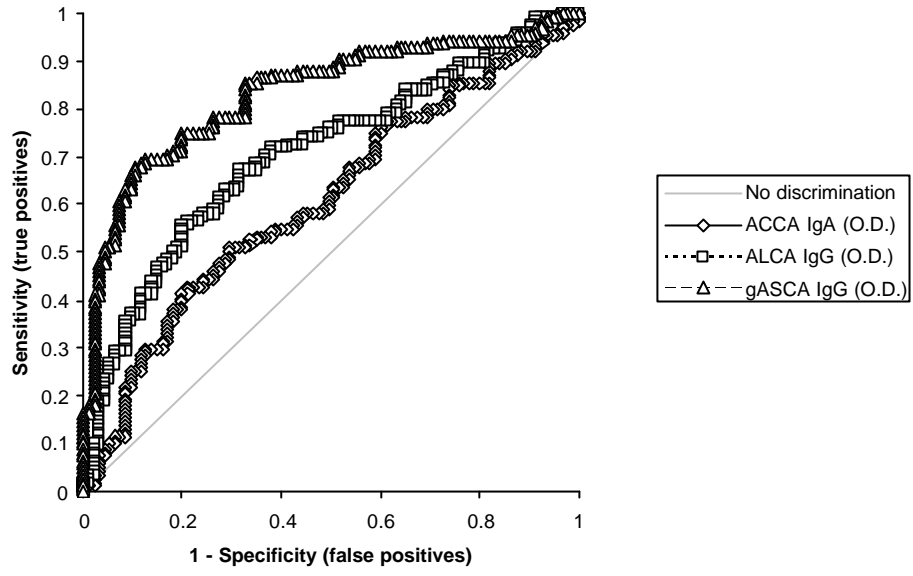


Table 1. Patient characteristics in the discovery screening.

	CD	UC	HC
	(n=72)	(n=56)	(n=41)
Age, years (range)	35.5 (19–70)	40.9 (14–89)	38.9 (18–78)
Female, n (%)	31 (43)	28 (50)	21 (51)
Mean disease duration, years (range)	9.1 (1–54)	7.9 (1–39)	–
Mean age at diagnosis, years (range)	26.6 (8–63)	33 (10–87)	–
Bowel surgery (including appendectomy), n (%)	28 (38.9)	3 (5.4) **	–
Extraintestinal manifestations, n (%)	28 (38.9)	14 (25.0)	–
Treatment:			
5-ASA, n (%)	48 (66.7)	53 (94.6)**	–
Antibiotics, n (%)	15 (20.8)	4 (7.1)	–
Steroids, n (%)	23 (31.9)	15 (26.7)	–
6MP/AZA, n (%)	22 (30.5)	12 (21.4)	–
MTX, n (%)	5 (6.9)	0 (0)	–
Infliximab, n (%)	9 (12.5)	0 (0) *	–
No treatment n (%)	0 (0)	0 (0)	–

* $p < 0.05$ versus CD

** $p < 0.0001$ versus CD

Table 2. Patient characteristics in the validation screening.

	CD (n=124)	UC (n=106)	OGD (n=61)	HC (n=40)
Mean age, years (range)	35.3 (16-71)	41.3 (14-89)*	33.7 (1-83)	37.0(18-78)
Female, n (%)	48 (39)	49 (46)	31 (50)	21 (51)
IBD patient characteristics				
Mean disease duration, years (range)	8.1 (1-54)	8.0 (1-39)		
Mean age at diagnosis, years (range)	26.9 (8-65)	32.1 (10-87)		
Bowel surgery (including appendectomy), n (%)	41 (33)	3 (2)*		
Extra intestinal manifestations, n (%)	47 (37)	22 (20)		
Smoking status:				
• Current and past smoker n (%)	57 (46)	36 (34)		
• Non-smoker n (%)	66 (53)	59 (56)		
• Unknown n (%)	1 (1)	11 (10)		
Origin:				
• Ashkenazi n (%)	65 (52)	52 (49)		
• Sephardic n (%)	46 (37)	23 (22)		
• Mixed n (%)	12 (10)	11 (10)		
• Non Jewish n (%)	0 (0)	8 (8)		
• Unknown n (%)	1 (1)	12 (11)		
CD characteristics:				
Disease location:				
• Small bowel n (%)	67 (54)	–		
• Colon n (%)	17 (14)	–		
• Both n (%)	40 (32)	–		
Disease behavior ^s :				
• Inflammatory n (%)	57 (46)	–		
• Stricturing n (%)	38 (31)	–		
• Penetrating n (%)	29 (23)	–		
Mean CDAI (SD)	185 (121)	–		

OGD group included 27 celiac disease patients, 20 irritable bowel syndrome (IBS) patients, and 14 patients with colonic polyps, diverticular disease, pseudomembranous colitis, *Helicobacter pylori* gastritis, pancreatitis, and hemorrhoids; ^s Disease behavior was classified based on the Vienna classification²⁴. CDAI Crohn's disease activity index;

* p<0.0001 versus CD.

Table 3. Glycans displayed on the Glyochip[®] glycan array.

Glycan number	IUPAC nomenclature	Glycan number	IUPAC nomenclature
1	Gal(β) 3-sulfate	25	GlcNAc(β 1,4)GlcNAc(β)
2	Gal(α)	26	GlcNAc(β 1,3)Gal(β 1,4)Glc(β)
3	Gal(β)	27	GlcNAc(β 1,6)GalNAc(α)
4	Gal(β 1,3)[GlcNAc(β 1,6)]GalNAc(α)	28	L-Rha(α)
5	Gal(β 1,3)GalNAc(α)	29	GalA(β)
6	Gal(β 1,3)GlcNAc(β)	30	Man(α)
7	Gal(β 1,4)Glc(β)	31	Man(α 1,3)Man(α)
8	Gal(β 1,4)GlcNAc(α)	32	Man(β)
9	Gal(β 1,3)Gal(β 1,4)GalNAc(β)	33	Man(β 1,4)Glc(β)
10	Gal(β 1-,6)Gal(β)	34	Neu5Ac(α)
11	GalNAc(α)	35	L-Araf(α)

Glycan number	IUPAC nomenclature	Glycan number	IUPAC nomenclature
12	GalNAc(β)	36	GlcA(β)
13	Fuc(α)	37	Xyl(α)
14	Fuc(α 1,2)Gal(β)	38	Xyl(β)
15	Fuc(β)	39	Gal(α 1,3)Gal(β 1,4)GlcNAc(β)
16	Glc(α)	40	Gal(α 1,3)Gal(β 1,4)GlcNAc(β 1,3)Gal(β 1,4)Glc(β)
17	Glc(α 1,4)Glc(α)	41	Gal(α 1,4)Gal(β 1,4)Glc(β)
18	Glc(α 1,4)Glc(β)	42	Glc(β 1,4)Glc(β)
19	Glc(β)	43	Man(α 1,2)Man α
20	Glc(β 1,3)Glc(β)	44	Man(α 1,3)[Man(α 1,6)]Man(β)
21	GlcNAc(β) 6-sulfate	46	Man(α 1,6)Man α
22	GlcNAc(α)	46	Mannan
23	GlcNAc(β)	47	Xylan
24	GlcNAc(β 1,3)GalNAc(α)	48	Dextran

Table 4: Anti-glycan IgG antibodies in IBD patients and healthy controls.

Glycan	Signal intensity, RFU*10 ⁶		
	Mean (SD)		
	CD (n=72)	UC (n=56)	HC (n=41)
Glc(b1,3)Glc(b)	2.26 (1.99)	0.91 (1.13)**	1.56 (1.39)*
laminaribioside			
a-Man	1.35 (0.70)	0.62 (0.30)**	0.62 (0.29)*
Man(a1,3)[Man(α1,6)]Man (β)	1.24 (0.84)	0.46 (0.18)**	0.53 (0.25) *
Man(a1,3) Man(a)	1.42 (0.98)	0.50 (0.22)**	0.66 (0.57) *
Mannan	4.91 (2.34)	1.91 (1.28)**	1.97 (1.28)**

Serum samples from 72 CD and 56 UC patients and 41 healthy controls were screened for the presence of anti-glycan antibodies using a glycan array. Of the 48 glycans screened, IgG antibodies to five glycans – mannan and mannan residues and laminaribioside – were significantly increased in CD compared with UC patients, and to four glycans in CD compared with healthy controls. Results are presented as mean ± SD.

* p <0.05 versus CD

** p <0.001 versus CD

Table 5. Anti-glycan IgA antibodies in IBD patients and healthy controls.

Glycan	Signal intensity, RFU*10⁶		
	Mean (SD)		
	CD (n=72)	UC (n=56)	HC (n=41)
GlcNAc(b 1,4)GlcNAc(b)	0.68 (0.48)	0.39 (0.30) **	0.50 (0.33)
Chitobioside			
Mannan	2.32 (1.89)	0.97 (0.22) **	1.27 (0.41) *

Serum samples from 72 CD and 56 UC patients and 41 healthy controls were screened using a glycan array. Of the 48 glycans screened IgA antibodies to two – mannan and chitobioside – were significantly increased in CD compared with UC patients. Results are presented as mean \pm SD.

* $p < 0.05$ versus CD

** $p < 0.001$ versus CD

Table 6. Differentiation between CD and UC patients based on ASCA, ALCA, ACCA and gASCA levels.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive Value (%)	Efficiency (%)
ASCA IgG	66.1	85.8	84.5	68.4	75.2
At least one positive of ALCA, ACCA, gASCA	77.4	90.6	90.6	77.4	83.5
At least two positive of ALCA, ACCA, gASCA	26.6	99.1	97.1	53.6	60.0
All three: ALCA, ACCA, gASCA are positive	3.2	100.0	100.0	46.9	47.8

Serum samples from 124 CD and 106 UC patients were tested for the presence of ASCA IgG or the anti-glycan antibodies ALCA ACCA, and gASCA using ELISA. Serum samples were considered as positive if OD values were above the following cut-off values: 0.43, 1.55, and 0.4 for ACCA, ALCA and gASCA, respectively. These cut-off values were determined using receiver operating characteristic (ROC) curves (see Figure 4) to provide 97%, 100%, and 92% specificity for ACCA, ALCA, and gASCA, respectively, for differentiation between CD and UC patients.

Table 7. Differentiation between CD patients and control (OGD and healthy controls) patients (n=101) based on ASCA, ALCA, ACCA, and gASCA levels.

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive Value (%)	Efficiency (%)
ASCA IgG	66.1	87.8	90.1	60.7	74.2
At least one positive of ALCA, ACCA, gASCA	77.4	70.3	76.2	71.7	74.2
At least two positive of ALCA, ACCA, gASCA	36.3	96.0	91.8	55.1	63.1
All three: ALCA, ACCA, gASCA are positive	8.1	99.0	90.9	46.7	48.9

Serum samples from 124 CD patients and 101 controls (OGD and healthy controls) were tested for the presence of ASCA IgG or the anti-glycan antibodies ALCA, ACCA, and gASCA . Serum samples were considered as positive if OD values were above the following cut-off values: 0.41, 1.23, and 0.4 for ACCA, ALCA and gASCA, respectively. These cut-off values were determined using receiver operating characteristic (ROC) curves (see Figure 5) to provide 95%, 91%, and 92% specificity for ALCA, ACCA, and gASCA, respectively, for differentiation between CD and OGD patients.

OGD; other-than IBD gastrointestinal diseases, included 27 celiac disease patients, 20 irritable bowel syndrome (IBS) patients, and 14 patients with colonic polyps, diverticular disease, pseudomembranous colitis, *Helicobacter pylori* gastritis, pancreatitis, and hemorrhoids.

Table 8 Anti-glycan antibody levels in CD patients with different disease localization.

Antibodies	Log10 OD		
	Disease location	Mean (SD)	
	Colon (n=17)	Small bowel (n=67)	Both (n=40)
ALCA IgG	-0.25 (0.68)	-0.07 (0.59) *	-0.01 (0.54) *
ACCA IgA	-0.49(0.49)	-0.58(0.51)	-0.54 (0.48)
gASCA IgG	-0.54 (0.69)	-0.21 (0.61) **	-0.22 (0.54) *

Anti-glycan antibody levels within the CD cohort (n=124) were determined and associated with disease localization. For CD patients with disease localization to small bowel, or both in small bowel and colon, ALCA, and gASCA levels were significantly increased compared with CD patients with disease localization to the colon only.

* p<0.05 *versus* colon

** p<0.0001 *versus* colon

Table 9. Anti-glycan antibody levels in CD patients with different disease behavior.

Antibodies	Log10 OD		
	Mean (SD)		
	Disease behavior	Penetrating or stricturing (n=67)	inflammatory (n=57)
ALCA IgG		-0.03 (0.23)*	-0.13 (0.28)
ACCA IgA		-0.51(0.24)	-0.55(0.18)
gASCA IgG		-0.20 (0.23)**	-0.33 (0.31)

Anti-glycan antibody levels within the CD cohort (n=124) were determined and associated with disease behavior. For CD patients with penetrating or stricturing disease behavior, ALCA, and gASCA levels were significantly increased compared with CD patients with inflammatory (non-stricturing, non-penetrating) disease behavior.

* p=0.04 *versus* inflammatory

** p=0.008 *versus* inflammatory

Table 10: Quartile analysis of the CD cohort for ALCA IgG, ACCA IgA and gASCA IgG and disease characteristics.

Quartile sum range (number of CD patients in the quartile sum range)	3-4 (n=18)	5-6 (n=26)	7-8 (n=33)	9-10 (n=34)	11-12 (n=13)	<i>p</i> trend value
Disease phenotype						
Small bowel disease (%)	55	79	88	97	92	0.003
Small bowel surgery (%)	22	36	33	38	30	ns
Penetrating or stricturing disease behavior [§] (%)	33	50	61	56	69	0.054

The CD population was subdivided into 4 quartiles by the level of immune response toward the three glycan antigens- laminaribioside, chitobioside, and oligomannan, determined by the levels of ALCA IgG, ACCA IgA and gASCA IgG, respectively. Each patient received a score according to its quartile (1-4). Quartile sums were calculated by the addition of each individual's quartile values for each glycan antigen (range 3-12). Patients in the quartile sum range of 3-4 had the lowest, while those in the range of 11-12 had the highest level of reactivity, toward all 3 antigens. Small bowel disease patients include patients with disease in the small bowel only and in both small bowel and colon. [§] based on the Vienna classification²⁴. ns, not significant.