ORIGINAL ARTICLE

Antiglycan Antibodies as Serological Markers in the Differential Diagnosis of Inflammatory Bowel Disease

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Background: The objective of the study was to evaluate the diagnostic accuracy of recently developed antiglycan serological tests in clinical practice for the diagnosis of Crohn's disease.

Methods: This study was a cohort analysis of both clinical and biochemical parameters of patients with diagnosed inflammatory bowel disease compared with those in a control population. Antiglycan antibodies were determined using commercially available enzyme immunoassays. The setting was the outpatient unit of the gastroenterology department of a large, tertiary-care referral academic hospital. Participants were 214 consecutive patients, enrolled over a 5-month period, including 116 with Crohn's disease and 53 with ulcerative colitis, as well as 45 with other gastrointestinal diseases and 51 healthy controls.

Results: Anti–*Saccharomyces cerevisiae* antibodies showed the best performance (54% sensitivity and 88%–95% specificity for Crohn's disease). Among patients with negative anti-*Saccharomyces* antibodies, 19 (34%) had high titers of at least another tested antiglycan antibody. Anti-*Saccharomyces* and anti-laminaribioside antibodies were associated with disease involving the small bowel and with penetrating or stricturing phenotype. Anti-laminaribioside was significantly higher in patients with a familial history of inflammatory bowel disease.

Conclusions: The new proposed serological markers are significantly associated with Crohn's disease, with low sensitivity but good specificity. About one third of anti-*Saccharomyces*-negative patients may be positive for at least 1 of those markers. Antiglycan antibodies appear to be associated with characteristic localization and phenotype of the disease.

(*Inflamm Bowel Dis 2008;14:645–651*)

Received for publication August 3, 2007; Accepted November 13, 2007.

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DOI 10.1002/ibd.20368

Published online 31 January 2008 in Wiley InterScience (www.interscience.wiley.com).

Key Words: Crohn's disease, ulcerative colitis, anti–*Saccharomyces cerevisiae* antibodies

Inflammatory bowel diseases (IBD), including mainly Crohn's disease (CD) and ulcerative colitis (UC), are chronic pathologies of still unknown origin. The diagnosis of IBD and the differentiation between CD and UC are usually made through evaluation of clinical, laboratory, radiological, endoscopic, and pathological features.

The hypothesis that IBD could derive from 1 or more autoimmune reactions was proposed in the 1950s and thereafter confirmed by the finding of circulating autoantibodies in these patients. The first autoantibodies identified were the anticolon antibodies,[1] which were abandoned because of their low specificity, followed by antipancreas,[2] antierythrocytes,[3] antiendothelium,[4] and anti-p40[5] antibodies. Immunohistochemical analysis of involved intestinal tissue helps in the understanding of a putative immune pathogenesis of IBD. Abundant local production of immunoglobulins (Ig) was found in inflammatory infiltrate, with specific isotype switching.[6] Although under physiological conditions IgA was the most secreted Ig in the intestinal mucosa, in IBD patients there was an increase in production of IgG (in particular IgG1) by B cells. Other autoimmune pathologies (e.g., systemic lupus erythematosus) are characterized by a specific increase in IgG1 production, which is more effective in complement activation than IgG2 and therefore is probably involved in disease onset and maintenance.

Currently, serological markers, namely, anti–neutrophil cytoplasmic antibodies (ANCAs) and anti–*Saccharomyces cerevisiae* antibodies (ASCAs), are the most employed for diagnosis and prognostic stratification of IBD. ANCAs are directed against antigens found mostly in azurophilic granules of neutrophils and have a sensitivity of about 60% and a specificity of about 80% in UC diagnosis. ASCAs recognize carbohydrate epitopes of phosphopeptidomannan, a 200-kDa glycoprotein of the *Saccharomyces cerevisiae* cell wall, and have been specifically associated with CD, with 40%–60% sensitivity and 80%–90% specificity.[7–9]

Recent studies[10–12] reported the identification and preliminary evaluation of 3 new antiglycan antibodies, called

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anti–laminaribioside carbohydrate antibodies (ALCAs), anti–chitobioside carbohydrate antibodies (ACCAs), and anti–mannobioside carbohydrate antibodies (AMCAs). These markers may emerge as noninvasive tools useful for the diagnosis, prognostic stratification, and better comprehension of IBD immunopathogenesis.

In a prospective study, we measured the concentrations of ASCAs, ALCAs, ACCAs, and AMCAs in patients with IBD, as well as in subjects with other alimentary tract disorders and in healthy donors taken as controls. Furthermore, we assessed the diagnostic accuracy of these markers (either alone or combined) in the differential diagnosis between CD and UC, CD and non-IBD, and IBD and other gastrointestinal diseases. Finally, the potential applications of antiglycan antibodies in prognostic stratification and disease management were investigated.

MATERIALS AND METHODS

Between September 2006 and January 2007, we enrolled 214 patients suffering from IBD or non-IBD gastrointestinal disorders seen at the outpatient unit of the gastroenterology department of a large, tertiary-care referral and teaching hospital.[13] Among them, 116 had previous diagnoses of CD and 53 of UC, confirmed by commonly accepted diagnostic criteria.[14] Mean disease duration in the CD and UC patients was 11.7 and 11.5 years, respectively. CD patients were divided into subgroups according to the involved site and behavior, according to the Vienna Classification.[15] In particular, disease localization was established on the basis of previously performed endoscopic, histological, and radiological investigations. Disease activity was defined according to recent pathological history, physical examination, and laboratory results, mainly considering inflammatory markers.[16] Perianal involvement was established on the basis of history of either fistula or abscess independently from objective inspection. The same approach was adopted to ascertain the occurrence of extraintestinal complications (arthritis, biliary calculosis, renal calculosis, erythema nodosus, spondylitis, primary sclerosing cholangitis). Concerning the surgical anamnesis, we considered only interventions related to the studied diseases, whereas pharmacological history was focused on either steroidal or immunosuppressor current therapy. Population features are shown in Table 1.

As controls, we recruited 45 patients with gastrointestinal disorders other than IBD (21 men, 45.6%; mean age 52.3 years, range 28–79 years), including 9 subjects with celiac disease, 6 with irritable bowel syndrome, 4 with colic diverticulosis; the remaining 26 presented with microscopic colitis, intestinal polyposis, chronic viral hepatitis, hepatic steatosis, chronic gastritis, peptic ulcer, gastroesophageal reflux disease, or pancreatitis. Fifty-one blood donors (29 men, 56.9%; mean age 44.5 years, range 23–66 years) from the same hospital were chosen as healthy controls. Written informed consent was obtained from each participant.

TABLE 1. Characteristics of the Study Population

	CD (n = 116)	UC (n = 53)
Male, n (%)	81 (69.8%)	34 (64.1%)
Mean age (range), years	46 (18–75)	47 (26–80)
Smokers, n (%)	70 (60.3%)	19 (35.8%)
IBD history, n (%)	11 (9.5%)	3 (2.6%)
Mean disease duration (range), years	11.7 (0–37)	11.5 (1–36)
Clinical presentation		
Medical, n (%)	80 (68.9%)	53 (100%)
Surgical, n (%)	36 (31.1%)	
Localization		
Ileal, n (%)	55 (47.4%)	
Colic, n (%)	11 (9.5%)	
Ileocolic, n (%)	41 (35.3%)	
Upper gastrointestinal, n (%)	9 (7.8%)	
Proctitis, n (%)		10 (18.9%)
Proctosigmoiditis, n (%)		10 (18.9%)
Left colitis, n (%)		9 (17.0%)
Subtotal colitis, n (%)		8 (15.1%)
Pancolitis, n (%)		16 (30.1%)
Clinical activity		
Remission, n (%)	87 (75.0%)	45 (84.9%)
Mild activity, n (%)	24 (20.7%)	5 (9.4%)
Mild-moderate activity, n (%)	3 (2.6%)	2 (3.8%)
Moderate activity, n (%)	2 (1.7%)	1 (1.9%)
Disease behavior		
Nonstricturing, nonpenetrating, n (%)	37 (31.9%)	
Stricturing, n (%)	43 (37.1%)	
Penetrating, n (%)	36 (31.0%)	
Perianal involvement, n (%)	23 (19.8%)	2 (3.8%)
Extraintestinal complications, n (%)	40 (34.5%)	18 (34.0%)
Dysplasia or neoplasia, n (%)	5 (4.3%)	10 (18.9%)
Surgical operations, n (%)	69 (59.5%)	4 (7.5%)
Steroidal therapy in the last 6 months, n (%)	41 (35.3%)	15 (28.3%)
Immunosuppression, n (%)	23 (19.8%)	10 (18.9%)

Blood samples, drawn by venipuncture, were centrifuged within 4 hours of collection and stored at −20°C until analysis. Antibody levels were determined using the specific ELISA IBDX kit (Glycominds LTD, Lod, Israel) for each antibody type. The result for each sample was calculated dividing the average optical density (OD) of the sample by the average OD of the calibrator, multiplied by the number of arbitrary units (AUs) assigned to the calibrator. Referred

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within-assays imprecision was less than 11%, whereas between-assays imprecision was less than 15%.

Data analysis was performed by MedCalc Version 7.6 (1993–2007 Frank Schoonjans). The diagnostic accuracy of the markers was evaluated using receiver operator characteristics (ROC) curve analysis. Comparison of data among patient groups was performed by analysis of variance, followed by the Student-Newman-Keuls test. The Student *t* test was employed to compare 2 groups of patients. P values < 0.05 were considered statistically significant.

RESULTS

ASCA levels found in CD patients were significantly higher than those in the other groups ($P < 0.05$; Fig. 1A). Interestingly, ASCAs were also significantly higher in IBD subjects than in non-IBD subjects. Concerning ALCAs, significant statistical differences were observed comparing CD and UC or CD and other gastrointestinal diseases, but not CD and healthy controls. Moreover, healthy donors had ALCA levels significantly higher than patients with non-IBD disorders ($P < 0.05$; Fig. 1B).

ACCA levels were significantly higher in CD, UC, and healthy controls than in diseased controls, who showed particularly low antibody titers. No statistically significant differences were found either between CD and UC or between CD and blood donors (Fig. 1C).

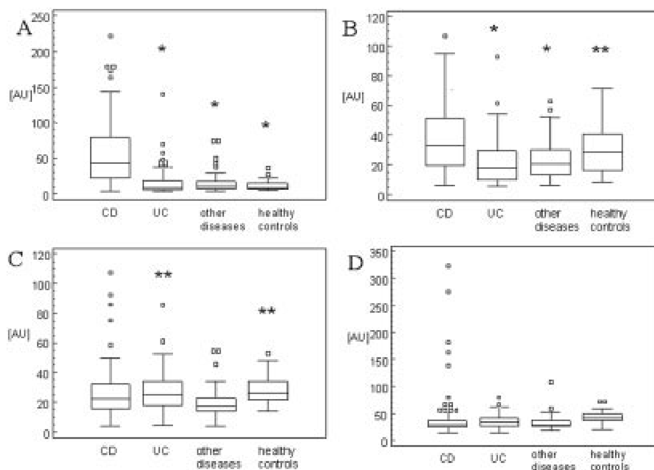

FIGURE 1. Distribution of antiglycan antibody titers among the 4 subgroups of studied patients. Nonparametric (notched box and whiskers, median, 95% confidence interval around the median, lower and upper quartiles and nonparametric percentile range) descriptive parameters are shown, indicating the central location and scatter/dispersion of the observations in our sample study. (A) Anti-*Saccharomyces cerevisiae* antibodies (ASCA). (B) Anti–laminaribioside carbohydrate antibodies (ALCA). (C) Anti–chitobioside carbohydrate antibodies (ACCA). (D) Anti–mannobioside carbohydrate antibodies (AMCA); $^{*}P < 0.05$ compared with CD group, $^{**}P < 0.05$ compared with controls with other diseases.

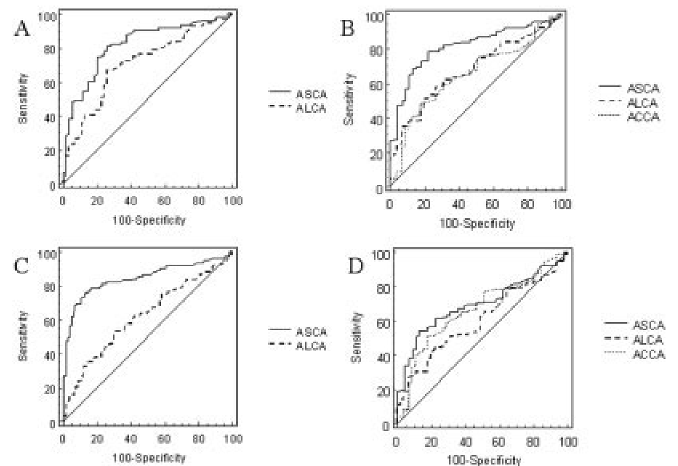

FIGURE 2. Receiver operating characteristic (ROC) curves of antiglycan antibodies. (A) Diagnostic accuracy of ASCA and ALCA in the differential diagnosis between CD and UC. (B) Diagnostic performance of ASCA, ALCA, and ACCA in discriminating between CD and other gastrointestinal disorders. (C) Diagnostic accuracy of ASCA and ALCA in differentiating CD patients compared with non-IBD conditions. (D) Diagnostic accuracy of ASCA, ALCA, and ACCA in the comparison of patients with IBD with those affected by other diseases.

AMCA concentrations were not significantly different among groups despite being on average higher in CD patients (Fig. 1D).

Based on differences in antibody titers found among groups, we calculated the accuracy of ASCAs and ALCAs in the differential diagnosis between CD and UC, between CD and other disorders, between CD and non-IBD, and between IBD and other gastrointestinal diseases. ACCA evaluation was restricted to the comparison between CD and other diseases, and IBD and other conditions. Because AMCA levels were not significantly associated with either a CD or an IBD diagnosis, they were not considered. ROC curves were subsequently obtained in order to evaluate the sensitivity and specificity of different markers in differential diagnoses. As shown in Figure 2, the ASCA ROC curve yielded the best diagnostic accuracy, with area under the curve (AUC) values of 0.818, 0.817, 0.846, and 0.704 for the considered differential diagnosis, respectively. For the same comparisons, ALCAs showed AUCs of 0.704, 0.677, 0.617, and 0.603, respectively.

According to the ROC curve analysis, cutoff values were calculated to consider whether each sample was positive or negative for ASCAs, ALCAs and ACCAs. For ASCAs (negative AU $\leq$ 40.3) and ALCsA (negative AU $\leq$ 50.5), the values chosen were similar to those proposed by the manufacturer. For ACCAs, because in our population the values were much lower than those reported by the manufacturer, a different threshold (negative AU $\leq$ 26.2) was adopted. To obtain high test specificity, we accepted low sensitivity val-

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TABLE 2. Diagnostic Accuracy of ASCAs, ALCAs, and ACCAs When Used as a Single Test

	ASCA				ALCA				ACCA			
	Se	Sp	PPV	NPV	Se	Sp	PPV	NPV	Se	Sp	PPV	NPV
CD versus UC	54.3	88.7	91.3	47.0	25.0	92.5	87.9	36.0	NS*	ND*	ND*	ND*
CD versus other diseases	53.4	91.1	94.0	43.6	25.0	93.3	90.6	32.6	38.8	88.9	90.0	36.0
CD versus non-IBD	54.3	95.8	94.0	63.4	25.0	88.5	72.5	49.4	ND*	ND*	ND*	ND*
IBD versus other diseases	40.8	91.1	94.5	29.1	19.5	93.3	91.7	23.6	40.2	88.9	93.2	28.4

Se, sensitivity (%); Sp, specificity (%); PPV, positive predictive value (%); NPV, negative predictive value (%); *not determined.

ues. Using these cutoff values, 63 of 116 CD patients (54.3%), 6 of 53 UC patients (11.3%), 4 of 45 patients with other alimentary tract disorders (8.8%), and no blood donors were considered ASCA positive. Among CD patients, we found 25.0% (26 of 116) ALCA positivity, whereas among UC and other gastrointestinal disease patients lower positivity rates (7.5% and 6.6%, respectively, corresponding to 4 of 53 and 3 of 45) were detected. ALCAs were positive in 15.7% of blood donors (8 of 51). Among ASCA-negative CD patients, 6 of 53 (11.3%) were positive for ALCAs, and 13 of 53 (24.5%) for ACCAs. Overall, 34% of ASCA-negative CD subjects were positive for at least 1 of the other 2 markers. Among patients with celiac disease, 11.1% (1 of 9) were ASCA positive, none was ALCA positive, and 22.2% (2 of 9) were ACCA positive. Altogether, 3 individuals with celiac disease were positive for at least 1 of the 3 tested markers.

In the subsequent analysis, the diagnostic accuracy of each marker when singularly used in the differential diagnosis was calculated (Table 2).

ASCA sensitivity for CD was 54.3%, with 88%–95% specificity in the differential diagnosis between CD and either UC, other diseases, or non-IBD patients. Positive predictive value (PPV) was high, whereas the lower negative predictive value (NPV) was dependent on the low sensitivity of the test. ALCAs yielded a sensitivity ranging from 19% to 25%, with a specificity similar to that for ASCAs. Where applicable, in the differential diagnosis in which their utilization were possible, ACCAs showed a 39%–40% sensitivity and 89% specificity.

To assess the concentrations of different antibodies in combination as continuous variables, for each sample, we calculated a *serologic score* of 0 to 1 based on the sample AU values for each antibody (Table 3).

A serologic score greater than 0.45 as the cutoff for a CD diagnosis gave better sensitivity than that found with ASCAs alone, with a slightly lower specificity and PPV. As expected, the more the score increased, the more the sensitivity decreased and the specificity increased, reaching 100% when the calculated score exceeded 1.34 (Table 4). It should be emphasized that in this evaluation, we did not consider ACCA results in calculating the serologic score in the differential diagnosis between CD and UC as well as between CD and non-IBD.

Among CD patients, AMCAs were significantly higher in women and in smokers than in men and in nonsmokers ($P = 0.02$ and $P = 0.03$, respectively). Concerning the role of family history, CD patients with at least an IBD-affected first-degree relative showed ALCA levels significantly higher than those without familial cases (59.8 versus 34.7 AU, respectively, $P = 0.0005$).

Considering the disease presentation, although we found on average higher ASCA levels in patients with surgical presentation than in those with medical presentation of CD, this difference did not reach statistical significance ($P = 0.06$). Ileal disease localization was associated with higher ASCA levels than colic disease localization ($P < 0.05$), whereas ALCAs showed a similar trend but without statistical significance ($P = 0.07$). Ileal disease localization was also associated with a higher serologic score ($P < 0.05$). Regarding disease behavior, higher ASCA levels were found in patients with stricturing or penetrating disease than in cases with nonstricturing, nonpenetrating disease (59.8 and 76.7 AU, respectively, versus 34.3 AU; $P < 0.05$). Once again, ALCA showed the same trend but without reaching statistical significance (38.6 and 42.7 AU, respectively, versus 30.5 AU; $P = 0.09$). The calculated serologic score also was

TABLE 3. Serologic Score as Computed According to Arbitrary Unit (AU) Values for Each Tested Assay

ASCA	AU $<$ 20	20 $<$ AU $\leq$ 25	25 $<$ AU $\leq$ 40	40 $<$ AU $\leq$ 60	60 $<$ AU $\leq$ 120	AU $>$ 120
ALCA	AU $\leq$ 20	20 $<$ AU $\leq$ 25	25 $<$ AU $\leq$ 40	40 $<$ AU $\leq$ 60	60 $<$ AU $\leq$ 75	AU $>$ 75
ACCA	AU $\leq$ 20	20 $<$ AU $\leq$ 23	23 $<$ AU $\leq$ 28	28 $<$ AU $\leq$ 35	35 $<$ AU $\leq$ 55	AU $>$ 55
Score	**0**	**0.25**	**0.34**	**0.45**	**0.9**	**1**

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TABLE 4. Diagnostic Accuracy of Computed Serologic Score

	Score > 0.45			Score > 0.79			Score > 1.34		
	Se	Sp	PPV	Se	Sp	PPV	Se	Sp	PPV
CD versus UC	66.4	84.9	90.6	47.4	90.6	91.7	24.1	96.2	93.3
CD versus other diseases	69.0	82.2	90.9	47.4	91.1	93.2	24.1	100.0	100.0
CD versus non-IBD	66.4	82.3	81.9	47.4	90.6	85.9	24.1	100.0	100.0
IBD versus other diseases	53.3	82.2	91.8	35.5	91.1	93.7	17.8	100.0	100.0

Se, sensitivity (%); Sp, specificity (%); PPV, positive predictive value (%).

higher in patients with stricturing or penetrating disease behavior ($P < 0.05$). Finally, on comparing patients who underwent at least a surgical operation with those who were never operated on, significantly higher ASCA levels in the former group (65.7 versus 45.4 AU, respectively; $P = 0.02$) were found.

DISCUSSION

In the present study, we evaluated the levels and diagnostic accuracy of ASCAs, ALCAs, ACCAs, and AMCAs in 3 patient groups (with IBD or not) and in healthy controls. ASCA and ALCA antibody titers were significantly higher in CD patients than in all the other groups (except for ALCA in blood donors). Moreover, we found significantly higher ACCA levels in patients with CD, in patients with UC, and in healthy controls than in individuals affected by other alimentary tract diseases but without statistically significant differences between CD and UC or between CD and healthy population. These findings differ from those published by Dotan et al,[10] who showed statistically different ACCA titers between CD and UC patients as well. However, the authors reported high mean ACCA levels in healthy controls that were not statistically different when compared to CD patients. According to the comparison analysis, we applied ACCA results in the differential diagnosis between either CD or IBD and other gastrointestinal disorders, but not between CD and UC or between CD and non-IBD. As far as AMCAs are concerned, there were no significant differences among the 4 studied groups. Regarding this issue, although 1 study[11] reported significant AMCA differences, another performed on a very large population did not.[12]

According to ROC curve analysis, we established cutoff limits and obtained sensitivity and specificity values similar to those previously reported in the literature. ASCA sensitivity ranges from 40.8% to 54.3% in the considered differential diagnosis, whereas other markers had a markedly lower accuracy, not sufficient to support their separate use as screening tests. On the other hand, specificity and PPV were always good, confirming the association between considered markers (except ACCA) and CD. Taking into account the low sensitivity associated with the application of each marker separately, as previously reported by Ferrante et al,[12] as well as according to the manufacturer's diagnostic kit instructions, we tried to combine the results into a serologic score. This approach, which concurrently evaluated all 3 antibodies as continuous variables, correlated with an increase in sensitivity of about 10% and parallel dimunition in specificity. These results were similar to those reported by Ferrante et al,[12] whereas in the study by Dotan et al,[10] considering positivity for at least 1 marker (among ASCAs, ALCAs, and ACCAs) for CD diagnosis, the authors obtained an increase in both sensitivity and specificity in the differential diagnosis between CD and UC. Because no significant statistical difference in ACCA levels between CD and UC patients was found, we could not confirm the latter report. Although a serologic score does not seem suitable for use in the clinical practice, better sensitivity and specificity become relevant when considering ASCA-negative CD patients. Among them, 18 of 53 (34%) were positive for at least 1 of the other 2 markers, representing a subgroup of subjects who would not have been detected using ASCA alone. In the study by Dotan et al,[10] this subset represented 26% of ASCA-negative CD patients. In our investigation, there were no differences in the parameters examined between ASCA-positive and ASCA-negative, ALCA-positive, or ACCA-positive subgroups. Therefore, although some observations supported the combined use of serological markers in IBD diagnosis, there is a need for a computed serological score selected in relation to costs as well as results in our population. On extending the study of the serologic profile to larger groups of patients, it may also be possible to highlight phenotypic differences among subgroups positive for different markers.

Among CD patients, we also investigated possible associations between antibody titers and history and clinical features. Interestingly, we found significantly higher ALCA levels in those with at least a first-degree relative affected by IBD. To our knowledge, this has not been previously reported and can be of interest when discussing pathogenetic significance of antiglycan antibodies in IBD. Moreover, this may represent a link with genetics among other factors. Previous

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findings from Belgium have described that CD patients with 1 or more caspase activation and recruitment domain 15 (*CARD15*) variants were more frequently ALCA positive ($P < 0.0001$) and had higher ALCA titers ($P = 0.003$).[17] Further work on this issue could help in clarifying whether both CD genotypes and phenotypes help in clinical management.

The literature about ASCAs reports a significant association of this marker with ileal disease localization and with stricturing and penetrating disease behavior, as well as with the need for surgery. Our data confirm this evidence. In fact, ALCAs demonstrate a trend similar to ASCAs for the association with ileal localization and stricturing or penetrating disease behavior even if, as opposed to the work of Dotan et al,[10] in both cases they did not reach statistical significance. Overall, these findings support the existence of an association between antibody level and disease severity. Moreover, the serologic score results appear to be significantly associated with the most aggressive disease phenotypes, suggesting that the combined use of such markers may be useful in identifying patients with a more severe disease.

It is fundamental to understand whether antiglycan antibodies play a role in IBD immunopathogenesis or are only an epiphenomenon, associated with disease activity and increased intestinal permeability. Although the association between ASCA and increased intestinal permeability has been investigated by several studies,[18–20] there is no report of an association between ASCA level and disease activity, as confirmed by our data for both ASCA and the new antiglycan antibodies (75% of CD patients were in a remission disease phase). ASCAs have also been referred to as frequently positive in patients with celiac disease (40%–60%),[21,22] suggesting the hypothesis of their origin from chronic inflammation of small bowel. In our study, although the celiac disease subgroup was small, only 1 of 9 patients positive for ASCAs and 3 positive for at least 1 of the 3 considered markers were found. On the whole, on the basis of the published studies, increased ASCA levels in CD patients do not seem to result from increased intestinal permeability but probably represent an early phenomenon, even preceding the clinical appearance of the disease, maybe determined by the genetic profile of each patient.[23–25] These data suggest a genetic basis for ASCAs and other antiglycan antibody positivity, although the studies are not concordant.

In conclusion, our findings confirm the association between some new antiglycan antibodies and CD, pointing to a correlation between antibody titers, IBD familiarity, disease localization, and behavior. The diagnostic application of these markers becomes particularly important in the group of patients with CD who are ASCA negative but positive for at least 1 of the other 2 markers. Further studies are needed to clarify the correlation between a combined complete serological profile, disease phenotype, and, above all, therapeutic response. We suggest including ALCA and ACCA assessment in the diagnostic algorithm of patients with abdominal pain and chronic diarrhea, taking advantage of their lack of invasiveness, reasonably low cost, and quite good reproducibility. Among antiglycan antibodies, the role of AMCAs should be better defined.

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