

Anti-*Saccharomyces cerevisiae* antibodies (ASCA) in Crohn's disease are associated with disease severity but not NOD2/CARD15 mutations

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SUMMARY

Anti-*Saccharomyces cerevisiae* antibodies (ASCAs) have been proposed as serological markers, which may differentiate Crohn's disease (CD) from ulcerative colitis (UC) and predict disease phenotype. Their importance in pathogenesis is unproven. We investigated the relationship between ASCAs, disease phenotype and NOD2/CARD15 genotype in CD and whether ASCAs were related to antibodies to other fungal proteins. Serum from 228 patients [143 CD, 75 UC, 10 with indeterminate colitis (IC)] and 78 healthy controls (HC) were assayed for ASCA. Antibodies (IgA, IgG) to other fungal proteins (*Fusarium* species ATC20334, Mycoprotein) were measured in the same samples using an in-house enzyme-linked immunosorbent assay (ELISA) assay. ASCAs were present in 57% of CD, 19% of UC, 30% of IC and 8% of HCs. ASCA-positive status was a predictor for CD with sensitivity of 57%, specificity of 87%, positive predictive value of 78% and negative predictive value of 68%. ASCA was associated with proximal (gastroduodenal and small bowel involvement) rather than purely colonic disease ($P < 0.001$) and with a more severe disease phenotype and requirement for surgery over a median follow-up time of 9 years ($P < 0.0001$). No associations with NOD2/CARD15 mutations were seen. There was no association between ASCA and antibodies to MP (IgA or IgG). These data implicate ASCA as a specific marker of disease location and progression in CD, emphasizing the heterogeneity within IBD.

Keywords ASCA Crohn's disease disease behaviour disease location NOD2/ CARD15

INTRODUCTION

The chronic inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are now common causes of gastrointestinal disease in the western world [1]. CD and UC may present with similar symptoms, but differentiation is based on clinical features, anatomical distribution and pathological findings [2]. However, in approximately 10% of cases of colonic IBD, indeterminate colitis (IC) is diagnosed, as clear distinction between UC and CD is not possible [3].

The aetiology of CD is unknown, although both host genetic susceptibility [4] and enteric flora [5] are implicated in the characteristic dysregulation of mucosal immunity. Recently, the NOD2/CARD15 gene [6,7] has been identified as an important determinant of susceptibility to CD. The NOD2/CARD15 protein is expressed in a variety of cell types [8–10] and recent data

identified bacterial peptidoglycan as its ligand [11,12]. Three common CD-associated NOD2/CARD15 single nucleotide polymorphism (SNP) variants have been identified in European and North American populations: SNP8 [arginine/tryptophan substitution at position 702 (R702W)]; SNP12 [glycine/arginine substitution at position 908 (G908R)] and SNP13 [a frameshift mutation and terminal Leucine truncation (1007fs)] [13]. A number of less common variants have been described, with clear heterogeneity in the importance of these variants between ethnic groups [14,15]. In our Scottish population of CD patients, we have described a lower allele frequency of NOD2/CARD15 variants than in other cohorts in Europe and North America [16]. There is also heterogeneity within CD in the importance of the NOD2/CARD15 contribution, with associations described between NOD2/CARD15 carriage, early onset disease [14], ileal involvement [17] and stricturing/fistulating disease [18]. Defining genotype-phenotype variation is important, but subclassification of CD has proved a great challenge [19], particularly as the disease behaviour changes with time in a large proportion of patients [20].

Antibodies to several specific antigens have been reported in the sera of patients with IBD. It was hoped that studies of such antibodies would provide either insight into disease pathogenesis

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and heterogeneity or putative serological markers to adjunct/replace current diagnostic protocols. Great interest has been shown in anti-*Saccharomyces cerevisiae* antibodies (ASCAs), associated first with CD in the 1980s [21]. These antibodies have a 60–70% prevalence in CD patients compared with 10–15% in UC and 0–5% in healthy control subjects [22–24]. Perinuclear antineutrophil cytoplasmic antibodies (pANCA) have been proposed as a marker for UC, with 60–80% prevalence compared with 10% in CD patients [25,26]. Previous reports have suggested that ASCA and pANCA may be of value in differentiating between UC and CD [22,23,27]. The high specificity (85–97%) of these antibodies has potentially important clinical applications [22–24] but the low sensitivity (50–70%), when used alone, rules out their use as clinical screening tools. The pathogenic significance of these antibodies has not been established and it remains unclear whether they arise due to tissue damage, increased permeability or the mucosal immune perturbation seen in CD.

Existing studies investigating the relationship between ASCA and *NOD2/CARD15* status in CD have produced conflicting results [3,18], which may reflect clinical and genetic heterogeneity between patient populations. This study aimed to examine the prevalence of ASCA in a Scottish population of IBD patients and healthy controls and to look for associations between ASCA, disease phenotype and *NOD2/CARD15* genotype in CD patients. Antibody responses to a novel mycoprotein antigen (MP) were also studied to evaluate whether a more general underlying defect in tolerance to fungal material is a factor in the immune response to *S. cerevisiae*.

MATERIALS AND METHODS

Patients and controls

The medicine/oncology subcommittee of the Lothian Research Ethics Committee approved the study protocol. Consecutive consenting patients with a definite diagnosis of IBD were

recruited from those attending the IBD clinic at the Western General Hospital, Edinburgh. Diagnoses were defined by standard criteria for CD and UC [2]. Patients with IC were those whose initial diagnosis of IBD could not be confirmed clearly as CD or UC, by clinical, endoscopic or histological methods. Healthy controls (HC) were spouses/friends of IBD patients invited to attend for venepuncture at the same time as patients. None of the controls had IBD. Demographics of the subject groups are given in Table 1. Following informed consent, separate blood samples were collected for *NOD2/CARD15* genotyping and for assessment of ASCA and MP in serum at a single time-point. Serum samples were stored at -70°C until analysis. Sera were available for 228 IBD patients and 78 HC. Retrospective data were collected by patient interview and case-note review, which comprised demographic details, ethnicity, family history, smoking status, anatomical distribution and dates of symptom onset, diagnosis and surgery. In addition, for CD patients, disease behaviour at diagnosis and latest follow-up was recorded and changes noted. All data were anonymized and entered into a Microsoft Access® database. CD patients were classed for age at diagnosis, disease location and behaviour at both diagnosis and latest follow-up according to the Vienna classification [28] and are shown in Table 2. Follow-up data were available on 123 CD patients, with a median follow-up of 9 years (range 1 month – 54 years).

ASCA enzyme-linked immunosorbent assay (ELISA) method

The Medizym ASCA Combi kit (Sterilab Sciences, Harrogate, UK) was used to measure ASCA IgG + IgA in patients' sera, according to the manufacturer's instructions. The optical density (OD) of the developed assay was measured using a spectrophotometric ELISA reader at 405 nm wavelength. Results were interpreted by calculating the binding index (BI) with the formula: $\text{BI} = \text{OD}_{\text{sample}} / \text{OD}_{\text{cut-off control}}$. The cut-off control, positive and negative control samples were provided in the kit. A BI of ≥ 1 was

Table 1. Demographic data for CD, UC and IC patients and for healthy controls

	Crohn's disease (<i>n</i> = 143)	Ulcerative colitis (<i>n</i> = 75)	Indeterminate colitis (<i>n</i> = 10)	Healthy controls (<i>n</i> = 78)	<i>P</i> -value
Sex					
Female/male	79/64	34/41	7/3	39/39	$\chi^2 = 3.36$ n.s.
Age at sampling					
Mean (s.d.)	42.3 (16.1)	48.4 (14.1)	41.1 (15.2)	41.3 (16.7)	<i>P</i> = 0.006*
Range	17–87	24–79	23–68	20–74	
Age of onset					
Mean (s.d.)	28.1 (14.0)	36.3 (14.4)	29.5 (15.0)	n.a.	<i>P</i> < 0.001*
Range	5–76	16–76	9–67		
Known family history**					
<i>n</i>	31	16	5	4	$\chi^2 = 17.3$
(%)	22%	21%	50%	5%	<i>P</i> = 0.001
Smoking status					
Current	32 (22%)	6 (8%)	5 (50%)	16 (20%)	$\chi^2 = 20.7$
Ex-smoker	67 (47%)	41 (55%)	2 (20%)	48 (62%)	<i>P</i> = 0.002
Never	42 (30%)	28 (37%)	3 (30%)	13 (17%)	
Unknown	2 (1%)			1 (1%)	

n.s.: Not significant. *Significantly higher in UC than all other groups (Kruskal–Wallis test); **some patients were adopted and had no knowledge of birth family. n.a.: Not applicable.

Table 2. Vienna classification of CD patients and corresponding ASCA status

Vienna classification	n (%)	Median ASCA BI	ASCA positive n (%)	P-value‡
Disease location at diagnosis				
Ileum (L1)	45 (31)	1.89	31 (68.9)	P = 0.002
Colon (L2)	55 (39)	0.82	21 (38.2)	
Ileocolon (L3)	21 (15)	1.33	14 (66.7)	
Upper GI (L4)	16 (11)	1.57	13 (81.3)	
Perianal only	2 (1)	0.94	1 (50.0)	
Unknown	4 (3)			
With perianal disease	42 (29)	1.34	29 (69.1)	P = 0.07
Without perianal disease	97 (68)	1.03	51 (52.6)	
Age at onset/diagnosis				
Less than 40 years (A1)	118 (83)	1.12	69 (58.5)	n.s.
Over 40 years (A2)	24 (17)	1.09	13 (54.2)	
Unknown	1			
Juvenile onset (0–16)	17 (12)	1.31	13 (76.4)	n.s.
Early adult onset (17–39)	102 (71)	1.09	57 (55.9)	
Late adult onset (>40)	24 (17)	1.09	13 (54.2)	
Disease behaviour at diagnosis				
Inflammatory (B1)	91 (64)	0.94	42 (46.2)	P = 0.003
Stricturing (B2)	12 (8)	1.69	10 (83.3)	
Penetrating (B3)	28 (20)	1.65	21 (75.0)	
Unknown	12 (8)			
Change in disease behaviour: diagnosis to latest follow-up				
Unchanged inflammatory (B1)	40 (28)	0.75	9 (23.7)	P < 0.0001
Inflammatory to stricturing (B1–B2)	16 (11)	0.98	8 (50.0)	
Inflammatory to penetrating (B1–B3)	33 (23)	1.64	25 (75.8)	
Unknown	2			
Need for surgery				
Yes	91 (64)	1.64	64 (70.3)	P < 0.0001
No	52 (36)	0.79	17 (32.7)	

‡Details of statistical tests in text. n.s.: Not significant.

considered to be a positive result, <1 was considered to be a negative result.

Mycoprotein antigen preparation and ELISA

Unless stated otherwise, all reagents were from Sigma-Aldrich, Poole, Dorset, UK. Freeze-dried mycoprotein (MP) from the fungus *Fusarium* species ATCC 20334 (*F. graminearum*) were supplied by Marlow Foods Ltd. (Stokesley, UK). MP was heated in distilled water for 10–15 min, pepsin (10 µg/ml in 0.1 M HCl) was added and incubated for 1 h at 37°C. The preparation was neutralized with alkali, the supernatant collected for ELISA use and stored at –20°C. The MP supernatant was diluted (1 : 100) in bicarbonate/carbonate buffer, pH 9.6, and 100 µl added to each well of a 96-well high-binding ELISA plate (Greiner) and incubated for 5 h at 22°C. Plates were washed three times in ELISA wash [0.9% saline (Baxter's), 0.05% Tween 20] and blocked with ELISA wash containing 10 µg/ml human haemoglobin and 1% adult bovine serum. Plates were incubated for 1 h at 37°C and washed again. Serum samples diluted (1 : 100) in ELISA wash containing 1% adult bovine serum, were added to the plates in duplicate against standard and quality control samples, which were selected previously by screening stored sera from patients under investigation at the Gastrointestinal Unit, Western General Hospital, Edinburgh. The standard sample was used to

construct a standard curve. Plates were incubated overnight at 4°C. Alkaline phosphatase-conjugated antibody, specific for either IgG or IgA, was diluted in ELISA diluent at 1 : 1000 and incubated at 22°C for 5 h. The assay was developed using p-nitrophenyl phosphate dissolved in diethanolamine buffer and measured by optical density on a spectrophotometric ELISA reader at 405 nm wavelength. Results were calculated from the standard curve.

Statistical analysis

Sensitivity was the probability of a positive ASCA in a patient with CD; specificity was the probability of a negative ASCA in a patient with UC or HC. The positive predictive value (PPV) was the probability of having CD and a positive ASCA. The negative predictive value (NPV) was the probability of having UC or HC and a negative ASCA. The χ^2 test was used for analysis of discrete variables. Mann–Whitney and Kruskal–Wallis tests were used to compare the quantitative ASCA BI results between patient groups. Multivariate analysis of ASCA status compared with other variables was carried out using a binary logistic regression analysis. Data were analysed using Minitab™ Statistical Software, version 13.32 (Minitab Inc., PA, USA) or GraphPad Prism® version 4 (GraphPad Software, San Diego, CA, USA) and considered significant at $P < 0.05$.

RESULTS

ASCA: marker of Crohn's disease?

ASCA BI were calculated and individuals defined as ASCA-positive (ASCA⁺) or -negative (ASCA⁻). For the CD patients, 81/143 (57%) were ASCA⁺ compared with 14/75 (19%) UC patients, 3/10 (30%) IC patients and 6/78 (8%) HC ($\chi^2 = 64.7$, $P < 0.001$). The sensitivity of ASCA⁺ status for CD was 57%, the specificity 87%, PPV was 78% and NPV was 68%. ASCA BI were significantly higher in CD compared with all other groups (Kruskal-Wallis, $P < 0.0001$, Fig. 1a).

ASCA status was compared between patients with isolated colonic CD and those with UC or IC. ASCA BI of patients with colonic CD were significantly higher than those with UC and IC (Kruskal-Wallis, $P = 0.0031$, Fig. 1b). There was a significant difference in the number of ASCA⁺ patients with colonic CD (21/55, 38%), UC (14/75, 18%) or IC (3/10, 30%), $\chi^2 = 6.155$, $P = 0.046$. For the IC patients, one ASCA⁺ patient was later re-classified as CD, but no other patient had diagnostic features of either CD or

UC, so were still classed as IC. The other two ASCA⁺ IC patients were classed as truly indeterminate or with CD-like disease. For the ASCA⁻ IC, two had CD-like disease, three had UC-like disease and two were truly indeterminate.

No associations were found between ASCA and sex, smoking status, family history or treatment with Infliximab or Azathioprine (data not shown).

ASCA: marker of Crohn's disease location?

ASCA was found to be a marker of proximal CD: 81% with upper GI disease (L4) and 68% of patients with disease involving the ileum (L1 or L3) at diagnosis were ASCA⁺ compared with 38% of patients with colonic disease ($\chi^2 = 15.2$, $P = 0.002$). ASCA BI of patients with colonic disease (L3) were significantly lower than those with more proximal CD [L1, L2 or L4 (Kruskal-Wallis, $P < 0.001$)].

Several patients (29%) had concomitant perianal disease. In comparison, patients with perianal disease gave a trend for higher ASCA BI values than those without (Table 2, Mann-Whitney, $P = 0.07$). An increased proportion of patients with perianal disease were ASCA⁺ ($\chi^2 = 3.26$, $P = 0.07$).

ASCA: marker of age of onset of Crohn's disease?

No association was found between age of onset of CD and ASCA status using 40 years as the criteria. Further comparison between juvenile onset (<17 years) and early adult onset (17–39 years, Table 2) still gave no significant difference.

ASCA: marker of Crohn's disease behaviour?

Comparison of ASCA BI with behaviour at diagnosis showed that those patients with stricturing (B2) or penetrating (B3) disease had significantly higher ASCA BI than those with inflammatory (B1) disease (Kruskal-Wallis, $P = 0.003$, Table 2).

Of those patients with inflammatory (B1) disease at diagnosis ($n = 91$), 40 were B1 at follow-up, while 49 patients had stricturing (B2) or penetrating (B3) disease at follow-up (Table 2). Patients who changed from B1 to B2 or B3 disease between diagnosis and follow-up had higher ASCA BI than those whose disease remained B1 (Kruskal-Wallis, $P < 0.0001$, Table 2). At diagnosis, 12 patients had stricturing disease and 28 had penetrating disease. At follow-up 4/12 remained with stricturing disease but eight had changed to penetrating disease. There was no significant difference between the ASCA BI of those who had B2 or B3 disease at both diagnosis and follow-up, and those who changed from B2 to B3 (Kruskal-Wallis, $P = 0.173$, data not shown).

ASCA: marker of genotype?

Genotypic data for the CD patients were available for the three common *NOD2/CARD15* gene variants (Table 3a). There were no differences between ASCA⁺ and ASCA⁻ patients in the number of variants, overall carriage rate or allele frequency. A non-significant trend was seen between carriage of two abnormal copies of the *NOD2/CARD15* gene and ASCA positivity (Table 3b, $\chi^2 = 3.285$, $P = 0.07$).

A multivariate binary logistic regression analysis was performed for ASCA status and the following variables: sex, family history, smoking status, genotype, disease location at diagnosis, age at onset, disease behaviour at diagnosis, disease behaviour at latest follow-up and requirement for surgery. Of these, more severe disease behaviour at latest follow-up was found to be significantly and independently associated with ASCA status when

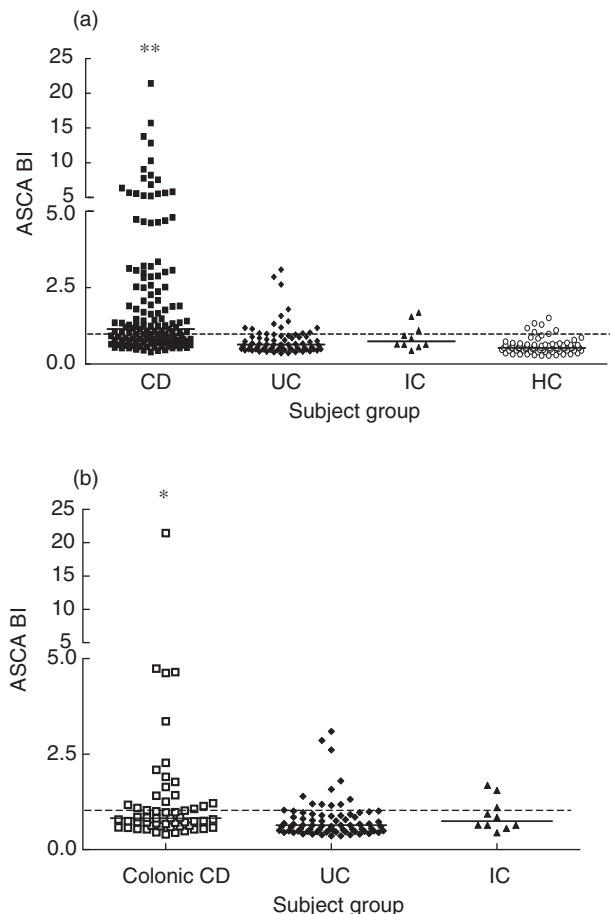


Fig. 1. ASCA as a disease marker. Column scatter plot of ASCA BI of patients in (a) all subject groups and (b) those with colonic CD, UC or IC. Dotted line denotes the ASCA BI cut-off value of 1. All values above the line are ASCA-positive. (a) Median ASCA BI: CD = 1.144, UC = 0.638, IC = 0.743, HC = 0.532. **ASCA BI of CD patients is significantly higher than all other groups ($P < 0.001$, Kruskal-Wallis). (b) Median ASCA BI: colonic CD = 0.824, UC = 0.638, IC = 0.743. *ASCA BI of colonic CD was significantly higher than that of UC or IC ($P = 0.0031$, Kruskal-Wallis).

Table 3. Analyses of ASCA status of CD patients by NOD2/CARD15 genotype

NOD2/CARD15 genotype*									
(a) ASCA status	<i>n</i>	Wild-type	Single heterozygous variant		Composite heterozygous variant				Homozygous variant
			<i>SNP 8</i>	<i>SNP 12</i>	<i>SNP 13</i>	<i>SNPs 12 & 13</i>	<i>SNPs 8 & 13</i>	<i>SNPs 8 & 12</i>	<i>SNP 8</i>
ASCA ⁺	81	65	5	2	2	3	0	1	3
ASCA ⁻	62	43	10	2	6	0	1	0	0

(b) ASCA status	<i>n</i>	Homozygous wild-type†	Single heterozygous variant	Composite heterozygous variant	Single homozygous variant	NOD2 variant allele frequency
ASCA ⁺	81	65 (80%)	9 (11%)	4 (5%)	3 (4%)	5.4%
ASCA ⁻	62	43 (69%)	18 (29%)	1 (2%)	0	4.7%

*No. of CD patients with each genotype. †No. (percentage) of CD patients in each genotype group.

all other factors were taken into account [$P = 0.001$, odds ratio = 3.21 (95% CI 1.58–6.52)].

Antibody responses to mycoprotein

The serum titres of both IgA and IgG against the MP antigen were measured and are shown in Fig. 2a,b. CD patients had significantly higher IgA titres than the other subject groups (Kruskal–Wallis, $P < 0.0001$, Fig. 2a). IgG MP titres were significantly higher in UC patients compared with other subject groups (Kruskal–Wallis, $P < 0.0001$, Fig. 2b). There was no correlation between ASCA BI levels and MP antibody titres (ASCA versus MP IgA, $r = 0.280$; ASCA versus MP IgG, $r = 0.049$). No correlation was found with disease location or phenotype in CD with either MP IgG or IgA, or with other demographic descriptors (data not shown).

DISCUSSION

In the present study we assessed the prevalence of ASCA in a Scottish cohort of IBD patients and healthy subjects and looked for associations with disease phenotype and NOD2/CARD15 genotype in the CD patients. A number of standardized ASCA assays have become commercially available, with differing degrees of specificity and sensitivity, although reconstructed receiver–operator curves have shown good concordance between different assays [29]. Thus our results can be compared directly with other studies, and the differences seen attributed to our population of patients rather than the methodology.

ASCA was found to be specific for CD, but of low sensitivity, which was in concordance with other studies [22–24]. Although ASCA BI levels were higher in patients with colonic CD than those with UC or IC, the considerable overlap between groups indicates that in isolation ASCA is not of great clinical usefulness in distinguishing between these patient groups. The concomitant use of pANCA and other serological markers [30] may aid in more easily differentiating between IC patients [23]. While these assays were not available for this study, it is in IC cases that ASCA/pANCA would perhaps be most useful to differentiate between the UC- or CD-like disease seen, as well as for possible further cat-

egorization of CD patients into clinically distinct subgroups, based on the ratio of the ASCA/pANCA levels measured [30,31].

Establishing phenotypic associations of CD subgroups may be important in terms of clinical practice and for the further understanding of disease pathogenesis. ASCA levels were raised in those patients who progressed to a severe disease phenotype and required surgery. In addition, ASCA was found predominantly in those CD patients with more proximal disease as others have shown [22,31–33]. Whereas Vasilaukas *et al.* stated that the presence, but not levels of antibodies were associated with disease location [31], in our patients, ASCA BI increased with proximity of disease. Disease location and behaviour are dynamic – changing over time [19,20]. In this study, ASCA status was determined on a single sample for each patient. If indeed, ASCA status is stable over time [34], regardless of disease activity or location, then a high ASCA BI may be indicative of future development of a severe disease phenotype once other contributory factors to ASCA levels have been taken into account, as borne out in the multivariate analysis. A longitudinal study of ASCA levels from diagnosis in patients whose disease behaviour changes would be important in confirming such relationships.

Does the presence of ASCA have a genetic basis? Although we found no relationship with familial CD, others have found associations, where both CD patients and unaffected family members have raised levels of antibodies compared with healthy controls [32,34,35]. These data gave rise to the hypothesis that ASCA may represent a specific, genetically determined breakdown in a mechanism of innate tolerance to yeast or fungal molecular patterns, to which the intestinal epithelium is exposed. Genetic linkage studies have not shown a linkage with the *IBD1* locus on chromosome 16 [13]. However, recent data have suggested that not only is there an association between *NOD2/CARD15* variants and ASCA, but that the presence of both is synergistic to produce fibrostenosing disease in paediatric CD [36]. In this study no association was found between ASCA and *NOD2/CARD15* variant carriage rate or allele frequency. Higher ASCA levels were seen in patients carrying two *NOD2/CARD15* variants, but this was not significant and may be due simply to the overall low allele fre-

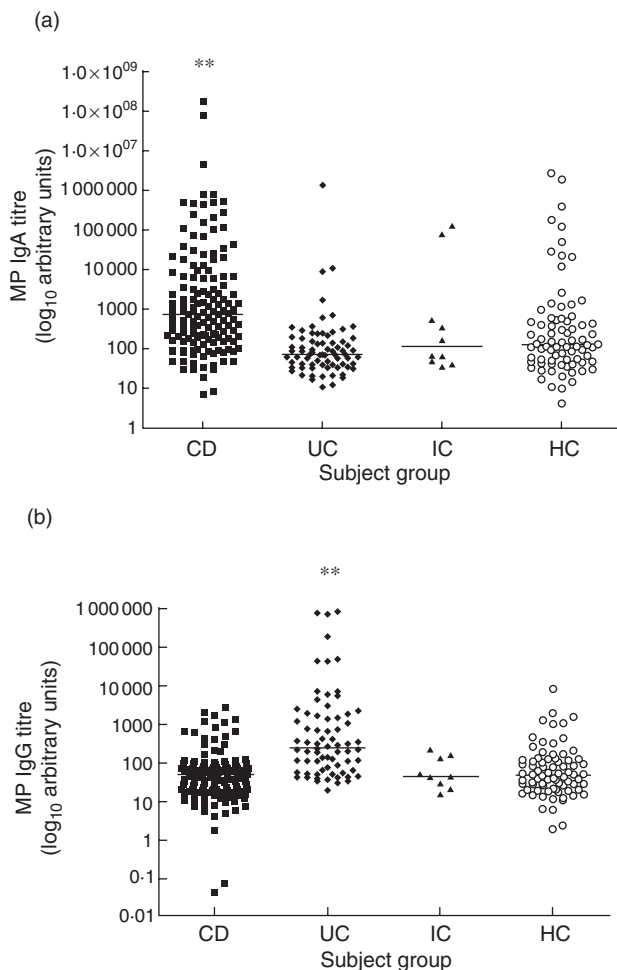


Fig. 2. Antibody titres to mycoprotein. Column scatter graphs of MP antibody titres for IgA (a) and IgG (b) in the different subject groups. Small bars denote median values. Antibody titres are in arbitrary units. (a) Median MP IgA titres: CD = 754, IC = 118, UC = 72.5, HC = 132. **MP IgA titre was significantly higher in CD compared with other subject groups ($P < 0.001$, Kruskal–Wallis). (b) Median MP IgG titres: CD = 50.4, IC = 44.0, UC = 238, HC = 47.2. **MP IgG titre was significantly higher in UC compared with other subject groups ($P < 0.001$, Kruskal–Wallis).

quency in our patients [16]. Indeed, our data strengthen the evidence for the lack of association of ASCA and *NOD2/CARD15* in that, despite a lower allele frequency of *NOD2/CARD15* variants, our population has comparable levels of ASCA. Other investigators also failed to find any such association, despite a 35% prevalence of *NOD2/CARD15* variants [18]. Similarly, ASCA seropositivity would not appear to be due to the downstream effects of *NOD2/CARD15* variants. Both *NOD2/CARD15* and ASCAs have been associated with ileal disease [18]; yet, in spite of this mutual association, independent mechanisms of CD pathogenesis must be acting in each case.

Patients with IBD have abnormal responses to food antigens [37,38], suggested to be secondary to inflammation and damage to the integrity of the intestinal wall, causing increased exposure of the immune system to antigenic contents of the bowel lumen [21], including *S. cerevisiae* antigens in baker's yeast. However, the highly specific association of ASCA with CD is hard to explain as such, and there is no correlation between ASCA and intestinal

permeability [34]. Thus the physiological importance of ASCA remains unclear. The presence of IgG ASCA-reactive antigens in the granulomas of bowel resections and on infiltrating lymphocytes and neutrophils in inflamed tissue from CD patients [39], are consistent with antigen specificity (or cross-reactivity) and suggest that *S. cerevisiae* itself may have some primary pathogenic role in CD. We hypothesized that a defect in immunological tolerance to *S. cerevisiae* may indicate a global loss of tolerance to fungal antigens, and so antibodies to MP were measured in our study. However, no correlation was found between ASCA and MP antibody titres. Surprisingly, MP IgA titres were higher in CD patients, while MP IgG titres were higher in UC patients. The importance of this observation is unclear; explanations may include differences in intestinal permeability or Th1/Th2 polarization of immune responses occurring in CD and UC.

In a similar way, the antibody class of ASCA may be important. While various studies have measured both IgA and IgG ASCA, their results were given the general term of ASCA⁺ [31,34,40]. It has been observed that a few patients have only IgG- or IgA-ASCA, but these levels tended to be lower than in those patients who had both antibody classes (possibly indicating a lower antigenic load?) and no association with any clinical grouping has been shown [31]. Therefore, in this study an assay to measure both together was used in our study, although in the light of the MP results this may need to be re-addressed. Oshitani *et al.* did look at IgG subclasses of ASCAs and found that whereas both IgG1 and IgG3 were additionally raised in Behçet's disease, only IgG4 was increased in both UC and CD patients, possibly as a result of the chronic inflammation that occurs in IBD [41].

While no correlation with MP was seen, in CD other antibodies have been identified against epitopes on bacterial antigens: outer-membrane porin C (OmpC) from *Escherichia coli* and the bacterial sequence I2 [30]. I2, present in the mucosa of CD patients with active disease, has been associated with *Pseudomonas fluorescens* [42]. For all these antigens, the actual epitopes to which they are produced have not been elucidated, but it is still possible that specific immunological defects could underlie the variation and severity of disease observed in different patients.

In conclusion, this study has confirmed that ASCA is specific for CD, although in isolation its low sensitivity will limit its use in clinical differentiation between IBDs. ASCA was a marker for proximal CD and for a more severe disease phenotype, but was not associated with *NOD2/CARD15* variants in our patient population. No correlation was found between degree of ASCA and MP antibody responses, providing further evidence that the ASCA response may be specific rather than secondary to mucosal injury. Further investigations, including the relationship with other genotypic markers may provide significant steps forward in the understanding of CD, in terms of pathogenesis and in the creation of diagnostic and predictive clinical models.

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