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Pattern recognition receptor and autophagy gene variants are associated with development of antimicrobial antibodies in Crohn's disease

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Abstract

Background—We sought to investigate whether variants in genes involved in bacterial sensing and autophagy (*NOD2, TLR5, IRGM, ATG16L1*) and the interleukin-23 signalling pathway (*IL12B, IL23R, STAT3*) were associated with development of antimicrobial antibodies in patients with Crohn's disease (CD).

Methods—A cohort of 616 CD patients from a tertiary referral hospital (Mount Sinai Hospital, Toronto) was evaluated. DNA was tested for three CD-associated *NOD2* variants (3020insC, G908R, R702W), variants in *IRGM*, *ATG16L1, IL12B, IL23R, STAT3,* and a *TLR5*-stop mutation. Serum was analyzed by ELISA for anti-*Saccharomyces cervesiase* (ASCA) IgG and IgA, anti-outer membrane protein C (anti-ompC), anti-Cbir1 flagellin, and anti-*Pseudomonas fluorescens* (anti-I2).

Results—*NOD2* 3020insC was associated with cumulative seroreactivity by quartile sum (p=0.003) and number of positive antibodies (p=0.02). *NOD2* G908R was also associated with quartile sum (p=0.05). Increased ASCA seropositivity was associated with *NOD2* 3020insC (odds ratio (OR)= 1.9, p=0.02) and G908R (OR=1.8, p=0.05), and *ATG16L1* T300A (OR=1.4, p=0.01) variants; ASCA positive patients had an increased cumulative number of *NOD2* 3020insC and *ATG16L1* T300A variants (p=0.007). *TLR5*-stop mutation abrogated development of anti-flagellin in a dominant-negative fashion (OR=0.5, p=0.009). The *IRGM* CD risk variant was associated with increased anti-flagellin seropositivity (OR=1.5, p=0.03). *IL12B, IL23R,* and *STAT3* variants did not contribute to development of anti-microbial antibodies.

Conclusions—Variants in innate immune genes involved in pattern recognition and autophagy but not the IL-23 signaling pathway influence antimicrobial seroreactivity in CD. In particular, the additive effect of *NOD2* 3020insC and *ATG16L1* T300A suggests a role for autophagy in development of ASCA.

Keywords

Inflammatory bowel disease; biomarkers; ASCA; genetics; NOD2

Introduction

Genome wide association studies have led to significant advances in our understanding of inflammatory bowel disease (IBD). In particular, the discovery of susceptibility genes

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involved in innate and adaptive immune pathways has contributed to the model of IBD pathogenesis. Positional cloning of the *IBD1* locus on chromosome 16 led to discovery of the first Crohn's disease (CD) susceptibility gene, *NOD2* (1, 2), which encodes a cytoplasmic pattern recognition receptor that recognizes the bacterial moiety dimuramyl peptide. Two groups have recently observed that *NOD2*'s product instructs autophagy (3, 4), an innate immune process controlled by two other CD susceptibility genes, *ATG16LI* and *IRGM* (5, 6); this suggests that autophagy is a central innate immune pathway involved in CD.

In 2000, Oppman *et al.*'s discovery of interleukin-23 (IL-23), which shares its p40 subunit with IL-12 (7), led to a reassessment of disorders once thought to be driven by IL-12, the canonical Th1 cytokine. Most previous work implicating IL-12 in inflammatory disorders, including IBD, had targeted the p40 subunit (8). Murine models subsequently suggested a key role for IL-23 driven immune responses, in particular via the Th17 lineage, in IBD pathogenesis (9, 10). The discovery of IL-23 pathway components as IBD susceptibility genes (8, 11–16) has further reinforced the importance of IL-23 driven inflammation in disease ontogeny. The IL-23 signaling pathway is orchestrated by a heterodimeric receptor encoded in part by *IL23R*, and downstream by the transcription factor STAT3 (17). Variants in *IL23R*, *STAT3*, and *IL12B* (encoding the p40 subunit) have all been implicated in CD (11, 12, 15).

It has been argued that IBD results in part from an innate immune deficit leading to adaptive immune hyperactivity against luminal antigens (17, 18), a model substantiated by the enrichment of anti-microbial antibodies found in CD (19). The greatest body of evidence surrounds anti-*Saccharomyces cervesiase* antibodies (ASCA). Despite its name, there is uncertainty surrounding the source of antigen driving ASCA response, with *Candida albicans* as a potential immunogen (20). A number of other antibodies enriched in CD have been more recently described including anti-cBir1 flagellin (anti-flagellin), anti-outer membrane protein C (anti-ompC), and anti-*Pseudomonas fluorescens* (anti-I2) (21).

The influence of IBD gene variants on development of anti-microbial antibodies is incompletely understood. A number of studies have suggested an association between *NOD2* variants and development of antimicrobial antibodies, especially ASCA (19, 22–25). Devlin *et al.* demonstrated that *NOD2*, but not *TLR2*, *TLR4*, or *TLR9* variants, was positively associated with cumulative seroreactivity against a panel of anti-microbial antibodies including anti-I2, anti-flagellin, anti-ompC and ASCA (19). Another study suggested that a CD protective *TLR5*-stop mutation was associated with decreased anti-flagellin response in healthy controls (26). Flagellin is the natural ligand of the *TLR5* product Toll-like receptor 5 (TLR5). However, to our knowledge no studies have examined the interaction between autophagy or IL-23 pathway genes and development of anti-microbial antibodies. In the present study, we investigated the association between CD gene variants involved in bacterial sensing and autophagy (*NOD2, TLR5, IRGM, ATG16L1*) and the IL-23 signaling pathway (*IL12B, IL23R, STAT3*), with the presence of anti-microbial antibodies in CD patients.

Methods

Patients

Serum and DNA were collected from a cohort of CD patients (n=616) recruited at Mount Sinai Hospital in Toronto, Canada. Diagnosis and classification of CD was made using standard clinical, endoscopic, and histologic criteria (27). Ethics approval was obtained from the Mount Sinai Hospital Research Ethics Board, and informed consent was obtained from

all subjects. Samples and data were anonymized. Clinical characteristics are summarized in Table 1.

Genotyping

DNA was extracted from venous blood of patients. Single nucleotide polymorphisms (SNPs) were genotyped using the Illumina Golden Gate platform (28). SNPs (NCBI SNP IDs in square brackets) included those in *NOD2* (3020insC [rs2066847]; R702W [rs2066844]; and G908R [rs2066845]), *ATG16L1* (T300A [rs2241880]), *IRGM* ([rs11747270]), *TLR5* (TLR5-stop [rs5744168]), IL23R ([rs11465804]), *STAT3* ([rs744266]), and *IL12B* ([rs10045431])(1, 2, 11, 14, 15, 26). Utilized SNPs including genotype distributions are summarized in the Supplementary Table. There was a failure rate of less than 5% in genotyping each locus, thus accounting for variation in the number of reported results by locus. SNPs/genes were chosen for analysis based on their previous associations with Crohn's disease and immunological function; *NOD2* and *TLR5* encode pattern-recognition receptors, *NOD2*, *ATG16L1*, and *IRGM* encode products involved in autophagy, and *IL23R*, *IL12B*, and *STAT3* encode components of the IL-23 signalling pathway (17, 26).

Serological analysis

Sera were analyzed for ASCA IgA and IgG, anti-flagellin (anti-CBir1), anti-ompC, and anti-I2 by enzyme-linked immunosorbent assay (ELISA) at Cedars-Sinai Medical Center in Los Angeles, as previous described (19, 29). Antibody levels are expressed in ELISA units (EU/mL) in relationship to established standards, derived from a pool of patient sera with well-characterized disease found to have reactivity to these antigens. Prevalence of the antimicrobial antibodies in the study population is included in Table 1.

Statistical analysis

Quartile sum scores were tabulated as a semi-quantitative measure of cumulative seroreactivity. Antibody levels were given a score of 1 to 4 based on their quartile within the distribution, 4 denoting the highest. ASCA IgA and IgG values were log-transformed and standardized, and the higher standardized unit was utilized for determination of ASCA quartile, as previously shown (19). Quartile sums of 4 to 16 were determined by adding scores from each of the four antibodies. The number of patients in each quartile appeared normally distributed (data not shown). Associations between SNPs and quartile sums were evaluated using linear regression, assuming an additive genetic model. In addition, the number of positive antibodies was used as a second measure of cumulative seroreactivity and analyzed in the same fashion.

For analysis comparing SNPs and specific antibodies, antibody levels were dichotomized using pre-specified cutoffs. ASCA IgG was considered positive if >40 EU/mL, ASCA IgA if >20 EU/mL, anti-ompC if >23 EU/mL, anti-flagellin if >30 EU/mL, and anti I2 if >20 EU/mL. Patients were considered ASCA-positive if either IgA or IgG was positive. Screening for associations between CD risk variants and antibody positivity was undertaken using an additive genetic model and univariate logistic regression. Associations discovered by this method were further analyzed using multivariate logistic regression (with age at diagnosis, and disease behaviour and location as co-variates), and comparing mean antibody titers by linear regression. These subsequent analyses were undertaken using the best-fitting genetic model (*i.e.* dominant or recessive), as shown in results section. Quantitative data is expressed as mean +/− standard error of mean. All analyses were undertaken using SAS 9.2 (SAS Institute Inc., Cary). Statistical significance was considered to be $P<0.05$.

Results

NOD2 3020insC is associated with cumulative seroreactivity to microbial antigens

Analysis confirmed a positive association between *NOD2* 3020insC and quartile sum (p=0.003) (Supplementary Table). The association between *NOD2* G908R and quartile sum also approached statistical significance (p=0.05). There was no association between quartile sum and *NOD2* R702W (p=0.85) or the other tested SNPs. A second surrogate measure of cumulative seroreactivity utilized was the number of positive antibodies. *NOD2* 3020insC (p=0.02), but no other tested SNP was associated with an increased number of positive antibodies.

NOD2 3020insC and ATG16L1 T300A variants are associated with increased ASCA seropositivity

There was a significant positive association between *NOD2* 3020insC and ASCA seropositivity (Odds ratio (OR)= 1.9 (95% confidence interval 1.1–3.2); p=0.02) (Figure 1A). *NOD2* G908R was also associated with ASCA seropositivity (OR=1.8 (1.0–3.4); p=0.05). In contrast, *NOD2* R702W (p=0.48) was not associated with ASCA. There was no association between *NOD2* variants and anti I2, anti-ompC and anti-flagellin in this cohort.

Like *NOD2* 3020insC, the *ATG16L1* T300A variant was associated with increased ASCA seropositivity (OR=1.4 (1.1–1.8); p=0.01) (Figure 1A), but not other antibodies.

Multivariate logistic regression, adjusting for disease location, behavior, and age at diagnosis, confirmed the independence of associations between *NOD2* 3020insC and ASCA (p=0.03, dominant model), and *ATG16L1* T300A and ASCA (p=0.02, recessive model).

To explore the cumulative effect of *NOD2* 3020insC and *ATG16L1* T300A on development of ASCA, a risk score was created; CD patients were stratified from 0 to 4 by number of *NOD2* 3020insC and *ATG16L1* T300A risk alleles. The risk score was significantly higher in ASCA positive patients (1.45 versus 1.24; p=0.007). There was an apparent additive effect of risk score on ASCA seropositivity (Figure 1B). Of note, all five patients homozygous for *NOD2* 3020insC and *ATG16L1* T300A were ASCA positive.

Analysis of NOD2 3020insC and ATG16L1 T300A variants and ASCA titers

Analysis comparing antibody titers was also undertaken to confirm the above associations. CD patients carrying at least one 3020insC variant (*i.e.* dominant model) had higher antibody titers of ASCA IgA (43.3 +/− 4.7 versus 26.3 +/− 1.3 EU/mL; p<0.0001) and IgG (65.0 +/− 6.2 versus 47.2 +/− 1.9 EU/mL; p=0.002); carriage of at least one G908R allele was only correlated with increased ASCA IgA levels (36.6 +/− 4.6 versus 27.4 +/− 1.4 EU/mL; p=0.03). However, *ATG16L1* T300A was not associated with elevated ASCA IgA or IgG titers (recessive model, not shown).

TLR5 and IRGM mutations are associated with anti-flagellin response

Using an additive genetic model, there was no significant association between *TLR5*-stop and anti-flagellin in the present study (p=0.10). However, this variant is known to act in a dominant fashion (30), and CD patients with at least one copy of *TLR5*-stop were less likely to be anti-flagellin positive (OR=0.51 (0.31–0.85); p=0.009 using dominant model) (Figure 2). The IRGM risk variant at rs11747270 (allele G) was associated with increased anti-flagellin seropositivity (OR 1.45 (CI 1.03–2.03); p=0.03). There were no significant associations between *IRGM* or *TLR5* variants and anti-flagellin titers or other antibody seropositivity.

IL-23 axis gene variants do not influence development of specific anti-microbial antibodies

Variants in *IL23R* (encoding a subunit of the IL-23 receptor), *IL12B* (encoding the p40 subunit shared by IL-23 and IL-12), and *STAT3* (encoding the downstream transcription factor STAT3) were not associated with antibody seropositivity (data not shown).

Discussion

Although previous work has investigated the molecular basis of the heritability of anti-microbial antibodies, it has mostly focused on the influence of pattern recognition receptors such as NOD2 and TLRs (19, 25). The present study explored the role of pattern recognition receptors, autophagy, and IL-23 signaling pathway genes on the humoral response against luminal antigens in Crohn's disease. We demonstrate that innate immune genes involved in pattern recognition and autophagy are important in development of ASCA and anti-flagellin in a cohort of CD patients.

Quartile sum analysis is commonly used as a measure of general antimicrobial seroreactivity in IBD; however, the fact that CD patients react to different subsets of antigens (21) suggests that this approach may obscure more specific relationships, in our case between gene variants and antibody development. In our cohort, only *NOD2* variants were associated with elevated quartile sums. No other tested gene variants appeared to influence this measure. Devlin *et al.* previously found that quartile sum was equally increased in patients with each of the three common *NOD2* variants (19). This difference is possibly a reflection of sample size (n=732 versus n=616) or population heterogeneity.

The two largest studies specifically investigating the heritability of antimicrobial antibodies (19, 25) both described the association between pooled *NOD2* variants, i.e. the presence of any *NOD2* variant and increased ASCA seropositivity. Neither reported on whether the ASCA association was driven by a specific *NOD2* variant. A smaller study (n=316) showed an association between G908R and 3020insC, but not R702W, and ASCA seropositivity (31). Our data suggests that the *NOD2* 3020insC mutation influences ASCA seropositivity and IgA/IgG titers. The G908R variant was also associated with ASCA seropositivity and IgA levels, though not at the same level of significance. We would suggest that the 3020insC variant, and to a lesser degree G908R, are largely driving the associations previously reported between pooled *NOD2* variants and ASCA.

It is becoming increasingly apparent that an inability of the innate immune system to recognize and clear intracellular bacteria is central to the pathogenesis of CD, supported by the association of CD with *NOD2*, *IRGM,* and *ATG16L1* polymorphisms (17). One model for understanding the development of anti-microbial antibodies in CD is that persistence of microbial products in the lamina propria because of defective clearance mechanisms results in loss of tolerance and hyperactive humoral response to microbial products. We therefore expected to find a broad association between *NOD2* polymorphisms and development of a number of antibodies, but instead found a predominant association with ASCA. It is not immediately apparent why a pattern recognition receptor recognizing the breakdown product of bacterial peptidoglycan (NOD2) would be principally associated with development of antibodies against a yeast polysaccharide (ASCA) versus bacterial antigens (anti-ompC, anti-flagellin, anti-I2). *NOD2* has no known role in recognition of *C. albicans* (32), an immunogen for ASCA (20). One possibility is that this effect is mediated by intestinal barrier dysfunction; the 3020insC mutation, but not R702W or G908R, has been associated with increased intestinal permeability (33). However, it has been suggested that ASCA does not result from defects in intestinal permeability (34, 35), and this possibility would furthermore not explain the prominence of the association between the 3020insC mutation and ASCA. We instead postulate that autophagy is an important mechanism controlling the

development of ASCA, supported by the association between *ATG16L1* T300A and ASCA, and the additive effect of *NOD2* 3020insC and *ATG16L1* T300A loci on ASCA seropositivity. Dendritic cells from CD patients with *NOD2* 3020insC or *ATG16L1* T300A variants are defective in both autophagy and presentation of antigen on MHC II (3). Autophagy defects may also influence development of anti-flagellin, as evidenced by the association described here between the IRGM CD risk variant and anti-flagellin seropositivity.

Following the observation that a dominant-negative *TLR5*-stop mutation abolishes TLR5 signalling (30), Gewirtz *et al.* demonstrated that this mutation reduces humoral response to TLR5's natural ligand, flagellin, in healthy controls, but not CD patients (26). They suggested that flagellin was likely dispensable as an adjuvant in the proinflammatory environment of CD. We demonstrated here, in a larger cohort of CD patients, that *TLR5*-stop is associated with reduced response to flagellin. It is appealing to suggest that flagellin acts as both antigen and adjuvant, inducing dendritic cell maturation via TLR5 (36) to orchestrate the anti-flagellin humoral response, and that loss of functional TLR5 thereby minimizes anti-flagellin immunoglobulin development in healthy and inflammatory (shown here) states. An additional hypothesis is that loss of functional TLR5 results in a change in composition of the intestinal microbiota, as has been shown in *TLR5*-deficient mice (37), and thus modulates the composition of luminal antigens.

We found no associations between any IL-23 signaling pathway gene polymorphisms and development of anti-microbial antibodies. A key action of IL-23 is in promoting immunopathology mediated by Th17 cells (38), a CD4+ T-cell subset important in mucosal proinflammatory responses. IL-23 driven pathways are classically invoked as an arm of the adaptive immune system(17). Within this paradigm, perhaps the association between innate but not IL-23 related genes and development of antimicrobial antibodies in CD is a reflection of Janeway's hierarchical model of innate instructing adaptive immunity (39). Nonetheless, it is becoming increasingly clear that IL-23 is a mediator of the intestinal innate immune system (40, 41).

In summary, we demonstrate in the present study that variants in innate immune genes involved in pattern recognition (*NOD2* and *TLR5*) and autophagy (*NOD2*, *ATG16L1*, and *IRGM*) influence antimicrobial seroreactivity in CD, in contrast to variants in the IL-23 signalling pathway (*IL12B*, *IL23R*, and *STAT3*). We confirm the previous association between *NOD2* variants and the presence of ASCA. Variants in *TLR5* and *IRGM* were associated with changes in anti-flagellin reactivity. *NOD2* and *ATG16L1* variants appeared to synergistically influence ASCA development. Future research should elucidate the role of autophagy in the development of antimicrobial antibodies in CD. Understanding the mechanism of generation of antimicrobial antibodies may shed light on their role in CD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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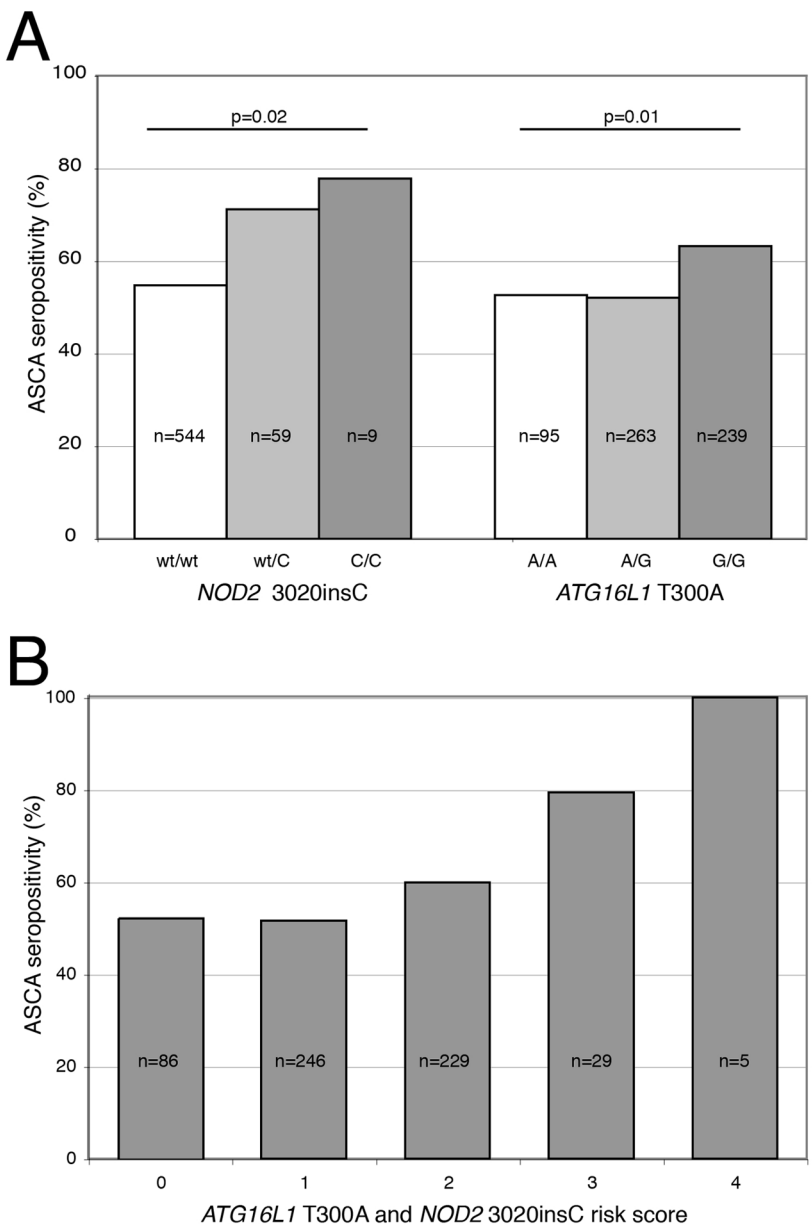

Figure 1.

(A) *NOD2* 3020insC (allele C) and *ATG16L1* T300A (allele G) are associated with increased ASCA seropositivity. P-value by logistic regression assuming additive genetic model. (**B)** ASCA seropositivity, stratified by 3020insC and *ATG16L1* T300A risk score

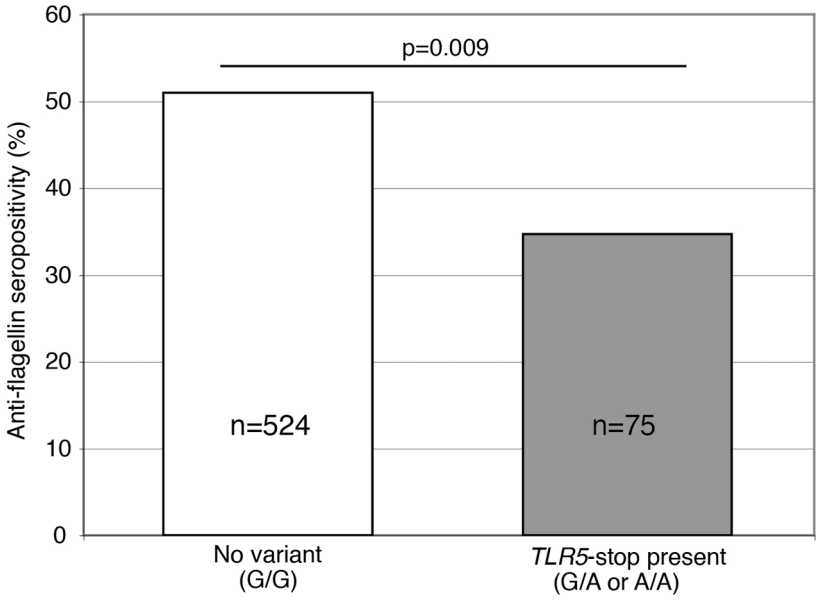

Figure 2.

TLR5-stop mutation (allele A) is associated with reduced anti-flagellin seropositivity in a dominant pattern in Crohn's disease patients.

Table 1

Clinical characteristics of the study population

Clinical characteristics	Crohn's disease subjects (n=616)
Male (%)	312 (50.6)
Median age at diagnosis in yrs (range)	19 (2 – 56)
Caucasian (%)	480 (77.9)
Disease location (%)	
L1 ileal	184 (30.5)
L2 colonic	119 (19.7)
L3 ileocolonic	300 (49.8)
Disease behaviour (%)	
B1 non-stricturing, non- penetrating	300 (49.4)
B2 stricturing	165 (19.7)
B3 penetrating	142 (49.6)
Prevalence of antibodies (%)	
ASCA IgA	264 (42.9)
ASCA IgG	283 (45.9)
anti-OmpC	190 (30.8)
anti-flagellin (CBir1)	302 (49.0)
anti-I2	310 (50.3)