Background and aims: An increased occurrence of anti-Saccharomyces cerevisiae antibodies (ASCA) is reported in unaffected members of families with Crohn’s disease. Whether ASCA is a familial trait due to genetic factors or is caused by exposure to environmental factors is unknown. To assess the genetic influence of ASCA we studied its occurrence in a twin population.

Patients and methods: ASCA were analysed in 98 twin pairs with inflammatory bowel disease and were related to clinical phenotype and CARD15/NOD2 genotype.

Results: ASCA were more common in Crohn’s disease than in ulcerative colitis (40/70 (57%) twins vs 5/43 (12%) twins). Associations with ileal Crohn’s disease, stricturing/penetrating behaviour, and young age, but not CARD15/NOD2 were confirmed. ASCA were found in 1/20 (5%) healthy siblings in discordant monzygotic pairs with Crohn’s disease compared with 7/27 (26%) in discordant dizygotic pairs. Using the intraclass correlation coefficient (ICC), no agreement in ASCA titres was observed in discordant twin pairs with Crohn’s disease, in monzygotic (ICC = −0.02) or dizygotic (ICC = −0.26) pairs. In contrast, strong agreement was seen within concordant monzygotic twin pairs with Crohn’s disease (ICC = 0.76).

Conclusions: These findings question the concept of ASCA as a marker of genetic susceptibility for Crohn’s disease. The agreement in ASCA titres within concordant monzygotic twin pairs with Crohn’s disease, suggests that the level of increase is genetically determined. We propose that ASCA are a marker of a response to an environmental antigen and that a specific gene(s) other than CARD15/NOD2 determines the level of response and perhaps also specific phenotypic characteristics.

METHODS

Twin

Twin pairs were derived from two Swedish cohorts of twins with IBD, both described previously.9–12 In brief, twin pairs where at least one twin in each pair had been hospitalised for IBD were identified by running the Swedish twin registry against the Swedish Hospital Discharge Register. The first cohort (n = 80 twin pairs) was identified in 1984 and the second cohort (n = 124 twin pairs) in 2000. A questionnaire was sent to all twins, including questions on diagnosis of IBD and general gastrointestinal symptoms. At the same time, consent from each twin to read his/her medical notes was requested. After responding to the questionnaire and obtaining written consent, the medical notes from twins with IBD or any history of gastrointestinal symptoms were scrutinised to verify or refute the diagnosis of IBD and to characterise the disease phenotypically. For CD, the Vienna classification was used.13 Discordant pairs refer to twin pairs where both twins are affected and in discordant pairs only one twin is affected.

Abbreviations: ASCA, anti-Saccharomyces cerevisiae antibodies; CD, Crohn’s disease; IBD, inflammatory bowel disease; UC, ulcerative colitis; ICC, intraclass correlation coefficient
Zygosity classification was based on the method applied by the Swedish twin registry. It relies on questions on childhood resemblance and has been shown to be very accurate. Zygosity classification was based on the method applied by the Swedish twin registry. It relies on questions on childhood resemblance and has been shown to be very accurate. A total of 151 twin pairs of the same sex, born between 1920 and 1980, with known zygosity, had approved further contact and were invited to take part in a study on ASCA.

ASCA

ASCA detection was performed as previously described. Briefly, antigens consisted of phosphopeptidomannan extracted from yeast cells of the S cerevisiae Su1 strain from cultures grown in bioreactors. Microtitration plates were coated with 100 μl of phosphoproteinmannan at a concentration of 1 μg/ml in sodium carbonate buffer (60 mmol/l, pH 9.6) for one hour at 37°C and overnight at 4°C, in moist chambers, and then washed four times in 50 mmol/l Tris HCL, 150 mmol/l NaCl, and 0.05% Tween 20 (TNT) (pH 7.5). Patients’ sera were diluted 1:101 in TNT and tested. After washing, 100 μl per well of a ready to use horseradish peroxidase labelled goat antihuman immunoglobulin (IgG, H and L chains; Zymed, Biosoft, Paris, France) were added, thereby detecting whole Ig ASCA, including both IgG, IgA, IgM; H and L chains; Zymed, Biosoft, Paris, France) were added, thereby detecting whole Ig ASCA, including both IgG and IgA. A colour reaction was obtained by using substrate chromogen (tetramethylbenzidine + H2O2) for horseradish peroxidase. Optical density was read at λ = 450/620 nm. Internal titrated standards were used for standardising the test. Results of individual sera were expressed in U/ml extrapolated from the standard curve. The cutoff was set at 7.2 U/ml.

CARD15/NOD2 polymorphisms

Data on CARD15/NOD2 status was available from two previous studies. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism in monozygotic twins17 and by polymerase chain reaction-sequence specific primers in dizygotic twins.18

Statistics

Differences in the presence of ASCA and other categorical data were analysed by Fisher’s exact test, adopted for small samples and for tables larger than 2×2, using StatXact version 6 (Cytel Software Corporation, Cambridge, Massachusetts, USA). Levels of ASCA titres were also evaluated in a quantitative multivariate perspective. Mean ASCA titres with corresponding 95% confidence interval (CI) for the different twin groups and estimates of the differences between the groups were analysed using regression analysis of a mixed model design with allowance for dependence within the twin pair, as well as with or without additional explanatory variables apart from those of diagnosis.19 Due to the complexity of the model and the limited number of twins, all clinical characteristics could not be included as explanatory variables. Location of CD (two categories—colon disease (L2) and small bowel involvement (L1+L3+L4)) and behaviour (two categories—non-stricturing non-penetrating (B1) and stricturing or penetrating behaviour (B2+B3)) were used to cross classify the CD twins into four different categories, all of which were then compared with the healthy twin of the CD patients. Smoking was introduced into the model (three categories—not smoking, ex-smoker, and smoker) but rejected due to statistical interaction with location and behaviour. To comply with the statistical assumptions, group comparisons were carried through on the logarthim of ASCA, and then the results were transformed back to the original ASCA scale. The effect parameter is therefore the ratio of geometric means rather than differences.20

To specifically address the question of agreement of ASCA titres within the twin pairs, we calculated the intraclass correlation coefficient (ICC) according to Dunn.22 This formulation of the ICC can be interpreted analogously to the weighted kappa index for agreement in paired measurements. A high value of ICC (≥0.80) indicates very good to excellent agreement whereas low ICC values (<0.40) indicate poor to fair agreement.23 It is possible with this formulation of ICC, which focuses on pairwise agreement, that severe disagreement might cause ICC to be negative. Comparisons of ICC for different twin groups may thus give insight into genetic and environmental influences.24

The Örebro County Ethics Committee approved the study.

RESULTS

Twins

A total of 151 twin pairs were invited to participate in the study. In 92 pairs (61%) both twins in each pair agreed to take part. Additionally, six twin pairs, known by us but not belonging to the epidemiological cohorts, also agreed to take part. Thus in total 98 twin pairs participated: 58 twin pairs with CD (concordant monozygotic n = 10, discordant monozygotic n = 20, concordant dizygotic n = 1, and discordant

<table>
<thead>
<tr>
<th>Twin individuals</th>
<th>ASCA+ (n = 40)</th>
<th>ASCA− (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40 y (A1)</td>
<td>55 (79%)</td>
<td>35 (50%)</td>
</tr>
<tr>
<td>&gt;40 y (A2)</td>
<td>15 (21%)</td>
<td>7 (10%)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terminal ileum (L1)</td>
<td>28 (40%)</td>
<td>20 (29%)</td>
</tr>
<tr>
<td>Colon (L2)</td>
<td>21 (30%)</td>
<td>6 (9%)</td>
</tr>
<tr>
<td>Ileocolon (L3)</td>
<td>19 (27%)</td>
<td>12 (17%)</td>
</tr>
<tr>
<td>Upper Gl (L4)</td>
<td>2 (3%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>Behaviour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-stricturing non-penetrating (B1)</td>
<td>30 (43%)</td>
<td>12 (17%)</td>
</tr>
<tr>
<td>Stricturing (B2)</td>
<td>21 (30%)</td>
<td>16 (23%)</td>
</tr>
<tr>
<td>Penetrating (B3)</td>
<td>19 (27%)</td>
<td>12 (17%)</td>
</tr>
<tr>
<td>CARD15/NOD2 genotype†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>56 (82%)</td>
<td>34 (50%)</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>12 (18%)</td>
<td>6 (9%)</td>
</tr>
<tr>
<td>Homozygote</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Data on CARD15/NOD2 genotype based on 68 twin individuals only.
†Arg702Trp, Gly908Arg, or Leu1007fsinsC.

Table 1  Clinical characteristics and anti-Saccharomyces cerevisiae antibody (ASCA) status of twins with Crohn’s disease according to the Vienna classification

Percentages refer to total number of twins (n = 70).

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ASCA in twins

Figure 1  Distribution of anti-Saccharomyces cerevisiae antibodies (ASCA) titres by pairs of twins. The broken lines correspond to the cut off values. Twin pairs are ordered along the Y axis according to decreasing ASCA titres on the left panel of each graph. In twin pairs concordant for the disease, the left panel comprises the twin in each pair with the highest ASCA titre and the right panel the twin with the lower ASCA titre. In twin pairs discordant for inflammatory bowel disease, the left panel comprises the diseased twin and the right panel the healthy twin sibling. (A) Monozygotic (MZ) twin pair with one twin suffering from Crohn’s disease (CD) and the other from ulcerative colitis (UC). (B) MZ twin pairs concordant for CD. (C) MZ twin pairs discordant for CD. (D) MZ twin pairs concordant for UC. (E) MZ twin pairs discordant for UC. (F) Dizygotic (DZ) twin pairs concordant for CD. (G) DZ twin pairs discordant for CD. (H) DZ twin pair concordant for UC. (I) DZ twin pairs discordant for UC.

ASCA status and titres in the different groups of twins

ASCA were detected in 40/70 (57%) twins with CD compared with 5/43 (12%) twins with UC, 8/47 (17%) healthy twin siblings to twins with CD, and 5/36 (14%) healthy twin siblings to twins with UC. There was a highly significant difference between the four groups (p<0.0001). Distribution of ASCA titres in each twin pair is shown in fig 1. Mean ASCA titre was 15.8 (95% CI 13.3–18.4) U/ml in CD twins compared with 4.2 (95% CI 0.7–7.6) U/ml in UC twins, 5.4 (95% CI 3.7–7.0) U/ml in healthy twin siblings to twins with CD, and 4.3 (95% CI 2.4–6.2) U/ml in healthy twin siblings to twins with UC. The difference between CD and UC twins was highly significant, the ratio of their means was 2.6 (95% CI
In ASCA titres (6.3 v 4.7 U/ml; p = 0.47), and the difference between CD twins and their healthy twin siblings was also statistically significant (ratio 2.2; 95% CI 1.8–2.7; p < 0.0001). UC twins and healthy twin siblings showed no difference (table 2). There was no increased occurrence of ASCA in healthy twin siblings in discordant monozygotic twin pairs with CD compared with dizygotic ones. Contrary to expectations, ASCA were found in 1/20 (5%) discordant monozygotic pairs and in 7/27 (26%) discordant dizygotic pairs (p = 0.11). However, no independent association was found between ASCA titres and zygosity in the multivariate analysis.

### ASCA status and titres according to CD phenotypes

**Location** Distribution of ASCA titres according to disease location is given in fig 2. ASCA were present in 6/21 (29%) twins with pure colonic CD (L2) and in 34/49 (69%) twins with small bowel involvement (L1+L3/L4) (p = 0.003). Similarly, the mean ASCA titre was 10.1 (95% CI 5.4–14.8) U/ml in twins with pure colonic CD (L2) and 18.2 (95% CI 15.2–21.1) U/ml in CD twins with small bowel involvement (L1+L3/L4) with a highly significant difference in the ratio of means (ratio 0.5; 95% CI 0.3–0.7; p < 0.0001) (table 2). Furthermore, ASCA titres in twins with small bowel involvement were higher than in their healthy twin siblings (ratio 3.0; 95% CI 2.3–3.8; p < 0.0001) (table 2). However, there was no significant difference between twins with pure colonic CD and their healthy twin siblings (p = 0.29) (table 2). There was no difference between healthy twin siblings of twins with pure colonic CD and their healthy twin siblings in ASCA status (2/17 v 6/30; p = 0.69) or in ASCA titres (6.3 v 4.7 U/ml; p = 0.47).

**Behaviour** Distribution of ASCA titres according to disease behaviour is given in fig 2. ASCA were present in 12/30 (40%) twins with non-stricturing non-penetrating CD (B1) and in 28/40 (70%) twins with complicated, either strictureting (B2) or penetrating (B3), CD (p = 0.016). Similarly, mean ASCA titres were lower in twins with non-stricturing non-penetrating CD (B1) (11.4 (95% CI 7.5–15.4) U/ml) than in twins with complicated disease (B2 or B3) (18.8 (95% CI 15.6–22.0) U/ml) with a ratio of 0.5 (95% CI 0.4–0.7; p = 0.0004). For comparisons of twins with CD and their healthy twin siblings, see table 2.

**Age** Distribution of ASCA titres according to age at diagnosis is given in fig 2. ASCA were present in 33/55 (60%) twins with age at diagnosis <40 years (A1) and in 5/15 (33%) twins with age at diagnosis >40 years (A2) (p = 0.04). Similarly, the mean ASCA titre was 18.9 (95% CI 16.1–21.8) U/ml in twins with age at diagnosis <40 years (A1) and 8.0 (95% CI 3.5–12.5) U/ml in twins with age at diagnosis >40 years (A2) (ratio 2.1; 95% CI 1.4–3.2; p = 0.0005) (table 2). Analysing ASCA in even younger CD twins (<30 years of age at diagnosis) did not change the results; ASCA were present in 27/43 (63%) and mean titre was 18.8 (95% CI 15.6–22.0) U/ml. For comparisons of twins with CD and their healthy twin siblings, see table 2.

### CARD15/NOD2

CARD15/NOD2 status was available in 68 of 70 CD twins. ASCA were equally distributed in CD twins with any of the three single nucleotide polymorphisms, Arg702Trp, Gly908Arg, or Leu1007fsinsC, as in CD twins with the wild-type genotype (6/12 (50%) v 33/56 (59%); p = 0.75) (fig 2). Mean ASCA titre was lower however, although not statistically significant, in twins carrying any of these polymorphisms (table 2).

### Multivariate perspective of phenotype ASCA association

As clinical characteristics in CD are dependent on each other, analyses with a quantitative multivariate perspective were added. In twins with pure colonic disease (L2) and a non-stricturing non-penetrating behaviour (B1), ASCA titres were similar to those in healthy twin siblings (table 3). In contrast, twins with either complicated CD behaviour (B2 or B3) or small bowel involvement (L1, L3, or L4) had significantly higher ASCA titres than their healthy twin siblings (p = 0.008 and p = 0.004, respectively).

### Agreement on ASCA titres within twin pairs

ASCA titres matched for each twin pair depending on zygosity, concordance, or discordance for IBD status are shown in fig 1. To assess the agreement of ASCA titres within twin pairs, the ICC was used. Greater agreement within monozygotic pairs with IBD than dizygotic ones (ICC = 0.44) was observed (table 4). Within concordant

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**Table 2** Ratio for anti-Saccharomyces cerevisiae antibodies (ASCA) mean between selected groups of twins. Estimates from mixed model with type of diagnosis as the only explanatory factor.

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Ratio of means</th>
<th>95% CI for ratio</th>
<th>p value for testing ratio = 1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-UC</td>
<td>2.6</td>
<td>1.9–3.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD-HT CD</td>
<td>2.2</td>
<td>1.8–2.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>UC-HT UC</td>
<td>1.0</td>
<td>0.8–1.3</td>
<td>0.73</td>
</tr>
<tr>
<td>HT CD-HT UC</td>
<td>1.2</td>
<td>1.0–1.6</td>
<td>0.10</td>
</tr>
<tr>
<td>CD colonic (L2)-CD small bowel (L1/3/4)</td>
<td>0.5</td>
<td>0.3–0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD colonic (L2)-HT CD colonic</td>
<td>1.2</td>
<td>0.9–1.7</td>
<td>0.29</td>
</tr>
<tr>
<td>CD small bowel (L1/3/4)-HT CD small bowel</td>
<td>3.0</td>
<td>2.3–3.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HT CD colonic-HT CD small bowel</td>
<td>1.1</td>
<td>0.8–1.6</td>
<td>0.47</td>
</tr>
<tr>
<td>CD non-strict non-penetr (B1)-CD strict/penetr (B2/3)</td>
<td>0.5</td>
<td>0.4–0.7</td>
<td>0.0004</td>
</tr>
<tr>
<td>CD non-strict non-penetr (B1)-HT CD non-strict non-penetr</td>
<td>1.4</td>
<td>1.0–1.9</td>
<td>0.03</td>
</tr>
<tr>
<td>CD strict/penetr (B2/3)-HT CD strict/penetr</td>
<td>3.1</td>
<td>2.4–4.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD &lt;40 y (A1)-CD &gt;40 y (A2)</td>
<td>2.1</td>
<td>1.4–3.2</td>
<td>0.0005</td>
</tr>
<tr>
<td>CD &lt;40 y (A1)-HT CD &lt;40 y</td>
<td>2.5</td>
<td>2.0–3.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD &gt;40 y (A2)-HT CD &gt;40 y</td>
<td>1.5</td>
<td>1.0–2.4</td>
<td>0.05</td>
</tr>
<tr>
<td>CD CARD15 mutated-HT CD CARD15 wild-type</td>
<td>0.66</td>
<td>0.4–1.1</td>
<td>0.09</td>
</tr>
<tr>
<td>CD CARD15 mutated-HT CD CARD15 mutated</td>
<td>1.67</td>
<td>1.0–2.9</td>
<td>0.07</td>
</tr>
<tr>
<td>CD CARD15 wild-type-HT CD CARD15 wild-type</td>
<td>2.37</td>
<td>1.9–3.0</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

CD, Crohn’s disease; UC, ulcerative colitis; HT CD, healthy twin sibling to twin with Crohn’s disease; HT UC, healthy twins to twin with ulcerative colitis; non-strict non-penetr, non-stricturing non-penetrating; strict/penetr, strictureting or penetrating; 95% CI, 95% confidence interval.
monozygotic twin pairs with CD, a high value was observed (ICC = 0.76). As there was only one concordant dizygotic pair with CD, ICC could not be calculated in this subgroup of twins. In both monozygotic and dizygotic twin pairs discordant for CD, low ICC values were observed (ICC = 0.02 and ICC = 0.26, respectively). Furthermore, in UC twin pairs, ICC was very high in discordant monozygotic but not in dizygotic pairs with UC (ICC = 0.84 and ICC = 0.21, respectively).

**DISCUSSION**

Based on family studies showing an increased occurrence of ASCA in healthy relatives, it has been proposed that ASCA may be a susceptibility marker for CD. Monozygotic twin pairs are genetically identical, in contrast with dizygotic twin pairs and siblings. However, in the present study, ASCA were found in only 1/20 healthy twin siblings in discordant monozygotic twin pairs with CD compared with 7/27 healthy twin siblings in discordant dizygotic pairs. Furthermore, no

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**Table 3** Analysis of anti-Saccharomyces cerevisiae antibodies (ASCA) in twins classified with Crohn’s disease (CD) in multivariate categories (stratification for location and behaviour)

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>Adjusted mean (95% CI) of ASCA (U/ml)</th>
<th>Ratio (95% CI) of ASCA for CD v healthy twin sibling; p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD, colonic (L2), non-strict non-penetr (B1)</td>
<td>14</td>
<td>4.8 (-2.6–12.2)</td>
<td>0.9 (0.6–1.5); p = 0.73</td>
</tr>
<tr>
<td>CD, colonic (L2), strict/penetr (B2/3)</td>
<td>7</td>
<td>17.1 (8.1–26.2)</td>
<td>2.2 (1.2–4.1); p = 0.008</td>
</tr>
<tr>
<td>CD, small Bowel (L1, 3, 4), non-strict non-penetr (B1)</td>
<td>16</td>
<td>16.0 (9.8–22.1)</td>
<td>2.2 (1.4–3.4); p = 0.004</td>
</tr>
<tr>
<td>CD, small Bowel (L1, 3, 4), strict/penetr (B2/3)</td>
<td>33</td>
<td>19.3 (15.0–23.6)</td>
<td>3.2 (2.4–4.4); p = 0.0001</td>
</tr>
<tr>
<td>HT CD</td>
<td>47</td>
<td>5.2 (3.2–7.2)</td>
<td>1.0 (reference)</td>
</tr>
</tbody>
</table>

CD, Crohn’s disease; non-strict non-penetr, non-stricturing non-penetrating; strict/penetr, stricturing or penetrating; HT CD, healthy twin sibling to twin with Crohn’s disease. Adjusted mean values of ASCA and ratios of ASCA mean between CD categories and healthy twins (reference category). 95% CI, 95% confidence intervals as well as parameter estimates.
No differences were found in either ASCA status (p = 0.09) or ASCA titres (p = 0.99) between UC twins and their healthy twin siblings. Interestingly, a high correlation for ASCA titres was also observed in discordant monozygotic UC pairs (ICC = 0.84) but not in discordant dizygotic twins (ICC = 0.21).

It has recently been hypothesised that relevant commensal bacteria can trigger and perpetuate a more complicated disease behaviour in genetically susceptible subjects. Furthermore, it has been proposed that immune responses are closer to the pathophysiological pathway of complicated disease behaviour than genetic susceptibility. However, concordance in ASCA titres in discordant monozygotic twin pairs with CD also suggests that genes determine the level of ASCA response.

The CARD15/NOD2 gene has been identified as an important determinant of susceptibility to CD. There are marked geographic differences in the occurrence of CARD15/NOD2 polymorphisms. The low frequency observed in the twin cohort is in accordance with data from other Scandinavian and Northern European countries, in contrast with other parts of Europe and North America. CARD15/NOD2 polymorphisms are associated with the same CD phenotype as ASCA—namely, ileal disease, young age at diagnosis, and possibly also strictureing CD (7). CARD15/NOD2 polymorphisms are independently associated with the presence of ASCA. This is however controversial and has not been replicated by others. We were not able to find any qualitative or quantitative association in the twin cohort, which suggests that other genes determine ASCA titres. Interesting data on the influence of the genetics of ASCA was recently reported. In a subgroup of CD patients carrying mutations in the exon of the mannan binding lectin gene, production of mannan binding lectin was low and those patients were more often ASCA positive.

In summary, no increased occurrence of ASCA was observed in healthy twin siblings in discordant monozygotic twin pairs. This questions the concept of ASCA as a marker of genetic susceptibility for CD and rather points towards ASCA as a marker of shared environment. A high degree of concordance in ASCA titres was observed in concordant monozygotic twin pairs with CD, suggesting that the level of increase is genetically determined. Based on these findings we propose that ASCA are a marker of a response to an environmental antigen and that a specific gene(s) other than CARD15/NOD2 determines the level of response and perhaps also specific phenotypic characteristics.

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Authors’ affiliations
J Halfvarson, G Järnerot, Division of Gastroenterology, Department of Internal Medicine, Örebro University Hospital, Örebro, Sweden
A Standaert-Vitse, B Sendid, T Jouault, D Poulain, Laboratoire de Mycologie Fondamentale et Appliquée, Inserm E360, Faculté de Médecine, CHU Lille, Lille, France
L Bodin, Statistical and Epidemiological Unit, Clinical Research Centre, Örebro University Hospital, Örebro, Sweden
A Duhamel, Centre d’Études et de Recherche en Informatique Médicale, Faculté de Médecine, CHU Lille, Lille, France
J F Colombel, Department of Hepatogastroenterology, Hospital Huriez, CHU Lille, Lille, France
C Tysk, Division of Gastroenterology, Department of Internal Medicine, and Department of Clinical Medicine, Örebro University Hospital, Örebro, Sweden

Conflict of interest: None declared.
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