Early development of stricturing or penetrating pattern in Crohn’s disease is influenced by disease location, number of flares, and smoking but not by NOD2/CARD15 genotype

E Louis, V Michel, J P Hugot, C Reenaers, F Fontaine, M Delforge, F El Yafi, J F Colombel, J Belaiche

Background: Crohn’s disease is a heterogeneous entity. Disease behaviour, characterised as stricturing, penetrating, or non-stricturing non-penetrating, is a clinically important phenotype as it is associated with complications and need for surgery. It has recently been showed that the behaviour of Crohn’s disease changes over the course of the disease. Aim: To assess the association between rapid development of a penetrating or stricturing pattern of Crohn’s disease and demographic and clinical characteristics as well as NOD2/CARD15 genotype. Patients and methods: A total of 163 patients with a firm diagnosis of Crohn’s disease and who had non-penetrating non-stricturing disease at diagnosis were studied. Various demographic and clinical characteristics as well as antisaccharomyces cerevisiae antibody status and NOD2/CARD15 genotype were documented in these patients. These characteristics were compared in subgroups of patients according to evolution of disease behaviour five years after diagnosis. Results: Five years after diagnosis there were 110 (67.5%) patients with non-stricturing non-penetrating disease, 18 (11%) with strictureing disease, and 35 (21.5%) with penetrating disease. In multivariate analysis, only disease location and number of flares per year were significantly discriminant between the three subgroups (p=0.0009 and 0.0001, respectively). Ileal location of the disease was associated with a strictureing pattern while a high number of flares was associated with a penetrating pattern. Active smoking was also associated with a penetrating pattern compared with a non-stricturing non-penetrating pattern only. Conclusions: Early development of strictureing or penetrating behaviour in Crohn’s disease is influenced by disease location, clinical activity of the disease, and smoking habit, but not by NOD2/CARD15 genotype.

Table 1 Vienna classification of Crohn’s disease

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>Location*</th>
<th>Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, &lt;40 years</td>
<td>L1, terminal ileum†</td>
<td>B1, non-stricturing non-penetrating</td>
</tr>
<tr>
<td>A2, &gt;40 years</td>
<td>L2, colon</td>
<td>B2, strictureing¶</td>
</tr>
<tr>
<td>A3, &gt;70 years</td>
<td>L3, ileocolon</td>
<td>B3, penetrating§</td>
</tr>
</tbody>
</table>

*Maximum extent at any time before first resection.
†Limited to the terminal ileum with or without spill over into the caecum.
‡Any disease location proximal to the terminal ileum regardless of additional involvement.
¶Occurrence of constant luminal narrowing (radiological, endoscopic, or surgical) with prestenotic dilatation or obstructive signs, without penetrating disease, at any time in the course of the disease.
§Occurrence of intra-abdominal or perianal fistulas, inflammatory masses, and/or abscesses at any time in the course of the disease. Perianal ulcers, but not skin tags, are also included.

Abbreviations: CD, Crohn’s disease; ASCA, antisaccharomyces cerevisiae antibodies; PCR, polymerase chain reaction.
We have recently shown that the behaviour of CD changes over the course of the disease. The majority of patients present with non-stricturing non-penetrating disease at diagnosis while after 25 years the majority harbour either a stricturing or penetrating pattern. This changing behaviour may still be determined by both genetic and environmental factors that may influence its inclination and speed of evolution. An association between a stricturing pattern and NOD2/CARD15 variants has recently been suggested, but the NOD2/CARD15 genotype was not uniformly found as an independent factor. The only environmental factor clearly associated with CD is active smoking. Smoking is significantly more frequent in CD than in healthy controls. Smoking has also been associated with more aggressive disease, but not with other clinical or biological characteristics, particularly CD behaviour. Besides smoking, the demographic and clinical characteristics of the disease, including treatments used, may influence its changing behaviour. Anti-saccaromyces cerevisiae antibodies (ASCA) represent the main serological marker associated with CD. ASCA have not been definitively associated with particular features of CD although an increased prevalence has been described in small bowel CD and higher levels have been suggested in disease with early onset, as well as fibrostenosing and internal penetrating disease behaviours. Overall, these data suggesting an association between NOD2/CARD15 or ASCA and disease behaviour are flawed by different definitions of disease behaviour and by the absence of consideration of evolution of this behaviour over time.

The aim of our work was to look for an association between evolution of CD behaviour, defined according to the validated Vienna classification, over the first five years after diagnosis on the one hand and NOD2/CARD15 genotype, smoking habit, ASCA, and various clinical and demographic characteristics on the other.

PATIENTS AND METHODS

Patients

A total of 163 patients with a firm diagnosis of CD according to the criteria of Lennard-Jones, classified as non-stricturing non-penetrating disease (B1) at diagnosis based on the Vienna classification, and regularly followed at our institution (CHU Liège, Clinique St Joseph Liège) were studied. Patients gave informed consent for the study. These patients formed part of the population in which we previously studied factors influencing early development of stricturing or penetrating Crohn’s disease. This information was regularly updated during follow up. We have recently shown that the behaviour of CD changes over the course of the disease. The majority of patients present with non-stricturing non-penetrating disease at diagnosis while after 25 years the majority harbour either a stricturing or penetrating pattern. This changing behaviour may still be determined by both genetic and environmental factors that may influence its inclination and speed of evolution. An association between a stricturing pattern and NOD2/CARD15 variants has recently been suggested, but the NOD2/CARD15 genotype was not uniformly found as an independent factor. The only environmental factor clearly associated with CD is active smoking. Smoking is significantly more frequent in CD than in healthy controls. Smoking has also been associated with more aggressive disease, but not with other clinical or biological characteristics, particularly CD behaviour. Besides smoking, the demographic and clinical characteristics of the disease, including treatments used, may influence its changing behaviour. Anti-saccaromyces cerevisiae antibodies (ASCA) represent the main serological marker associated with CD. ASCA have not been definitively associated with particular features of CD although an increased prevalence has been described in small bowel CD and higher levels have been suggested in disease with early onset, as well as fibrostenosing and internal penetrating disease behaviours. Overall, these data suggesting an association between NOD2/CARD15 or ASCA and disease behaviour are flawed by different definitions of disease behaviour and by the absence of consideration of evolution of this behaviour over time.

The aim of our work was to look for an association between evolution of CD behaviour, defined according to the validated Vienna classification, over the first five years after diagnosis on the one hand and NOD2/CARD15 genotype, smoking habit, ASCA, and various clinical and demographic characteristics on the other.

ASCA status was defined in 90 patients for whom we had a serum sample dating back to the time of diagnosis, by commercial ELISA (Medipan Diagnostica, Selchow, Germany), according to the manufacturer’s instructions. Investigators who determined ASCA were blinded to the behaviour of the disease. Patients were considered as ASCA+ when either ASCA IgA or IgG was present.

The number of flares per year was evaluated retrospectively based on patient medical notes. A flare was considered when the physician in charge of the patient (EL, FF, MD, JB) documented it in the notes and changed the treatment accordingly. As patients had exclusively non-stricturing non-penetrating disease, these flares were characterised by a combination of digestive and general symptoms, including diarrhoea, abdominal pain, fever, weight loss, worsening of the general physical condition, or systemic manifestations. The number of flares was calculated until the development of strictureing or penetrating disease or over the first five years of the disease for patients who remained non-stricturing non-penetrating. A mean number of flares per year was then calculated for every patient. The information in the medical notes was available and judged to be reliable for 126 patients.

The number of steroid courses was also determined retrospectively from patient medical notes. As for flares, the number of steroid courses was calculated until the development of strictureting or penetrating lesions or over the first five years of the disease for patients remaining non-stricturing non-penetrating. A mean number of steroid courses per year was then calculated for every patient. The information in the medical notes was available and judged to be reliable for 126 patients.

Reliable information on immunosuppressive treatment was available in 145 patients. Only 24 patients had taken azathioprine during the first five years of the disease. Sixteen had started azathioprine after the development of penetrating disease. The remaining eight patients received azathioprine because of steroid dependent or chronic active disease. In these patients, the number of months of azathioprine treatment before the development of a penetrating or stricturing disease or over the first five years of the disease for those who remained non-penetrating non-stricturing was calculated.

Demographic and clinical characteristics of the patients are shown in table 2.

NOD2/CARD15 genotyping

In 98 patients, DNA was collected and genotyped for the three main variants of NOD2/CARD15 that are associated with CD (OMIM 605956), as previously defined. In this initial report, the nomenclature of mutations was derived from the sequence of IB1D. This sequence is identical to the smaller open reading frame of NOD2/CARD15 described by Ogura and colleagues. However, an alternative open reading frame, characterised by a translation initiation site located 81 nucleotides above the smaller one, was also reported by Ogura et al. Others have recently proposed this sequence as the sequence-reference for mutation annotation. In order to avoid confusion, we therefore used this new method of annotation in the study, which is easily deduced from the initial one by adding 27 to the initial number of the mutated amino acid.

The missense mutation R702W (ex R675W) (SNP8, GenBank accession No G67950) was genotyped by an allele specific polymerase chain reaction (PCR) procedure. After amplification (primers: 5'-ATC TGA GAA GCC CCT GCT CC-3' (wild-type, forward), 5'- ATC TGA GAA GCC CCT GCT CF-3' (mutated, forward), and 5'-CCC ACA CTT AGC CCT GAT G-3' (reverse), annealing temperature 58°C, 30 cycles), the PCR products were loaded on a 2% agarose gel with internal controls. Genotypes were directly deduced from the migration profiles. The missense mutation G908R (ex G881R) (SNP12,
Association between evolution of CD behaviour (B1, B2, or B3) and demographic, clinical, and biological characteristics: univariate analysis

In univariate analysis, disease location, number of flares per year, number of steroid courses per year, and ASCA status were significantly different between the three groups, while smoking and familial form were borderline for significance (table 3). There was no association between NOD2/CARD15 genotype (defined as variant carriers or not, or as wild-type, simple heterozygotes, compound heterozygotes, and homozygotes) and evolution of CD behaviour (table 3). There was no association between various NOD2/CARD15 variants and evolution of CD behaviour.

Association between evolution to subtypes of penetrating disease (B3) and demographic, clinical, and biological characteristics: univariate analysis

When intra-abdominal and perianal penetrating disease were analysed separately, significant differences were found. Location at diagnosis differed significantly between these groups, with proportions of L1, L2, L3, and L4 being 83.3%, 8.3%, 8.3%, and 0%, and 13.3%, 40%, 40%, and 6.7% in intra-abdominal and perianal penetrating disease, respectively (p=0.004). When perianal penetrating diseases were excluded from the group of penetrating disease (B3), the association between location of disease and disease behaviour was still significant (p<0.0001). Smoking was significantly more frequent in perianal (86.7%) than in intra-abdominal (45.5%) penetrating disease (p=0.03). However, the association between smoking and evolution of disease behaviour was still borderline significant when perianal penetrating disease was excluded from the group of penetrating disease (p=0.057). No other significant difference was found between intra-abdominal and perianal penetrating disease. As regards the association between evolution of disease behaviour and clinical, demographic, or biological characteristics, when those with perianal penetrating disease were excluded from the group of penetrating disease, apart from location of disease and smoking habit, the association also remained significant for the number of flares per year (still higher in penetrating disease (p=0.001)) while there was no significant association for the other parameters.

Association between evolution of disease behaviour (B1, B2, B3) and demographic, clinical, and biological characteristics: multivariate analysis

In multivariate analysis, only location of disease and number of flares per year were selected when comparing the three groups (p values for these parameters in the model were 0.0009 and 0.0001, respectively; the p value of the model was 0.0001). When patients who became B2 were compared with those who remained B1, only disease location was selected (p value of the model 0.0001). When patients who became B3 were compared with those who remained B1, both the number of flares per year and smoking were selected (p values of these parameters in the model were 0.0001 and 0.02, respectively; the p value of the model was 0.0001). Finally, when patients who became B2 were compared with those who became B3, the number of flares per year, location, and existence of a familial form of the disease were selected (p values of these parameters in the model were 0.0004, 0.001, and 0.04, respectively; the p value of the model was 0.0001).
Factors influencing early development of strictureing or penetrating Crohn’s disease

Table 3  Demographic, clinical, and biological parameters depending on Crohn’s disease behaviour*

<table>
<thead>
<tr>
<th></th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (%) (n=163)</td>
<td>n=110</td>
<td>n=18</td>
<td>n=35</td>
<td></td>
</tr>
<tr>
<td>&lt;40 years (A1)</td>
<td>82.0</td>
<td>88.9</td>
<td>94.3</td>
<td>0.18</td>
</tr>
<tr>
<td>&gt;40 years (A2)</td>
<td>18.0</td>
<td>11.1</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Location of the disease (%) (n=163)</td>
<td>n=110</td>
<td>n=18</td>
<td>n=35</td>
<td></td>
</tr>
<tr>
<td>Ileal (L1)</td>
<td>38.2</td>
<td>89.9</td>
<td>45.7</td>
<td>0.0003</td>
</tr>
<tr>
<td>Colonic (L2)</td>
<td>33.6</td>
<td>0</td>
<td>22.9</td>
<td></td>
</tr>
<tr>
<td>Ileocolonic (L3)</td>
<td>23.6</td>
<td>0</td>
<td>28.6</td>
<td></td>
</tr>
<tr>
<td>Upper gastrointestinal tract (L4)</td>
<td>4.6</td>
<td>11.1</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Sex (%) (n=163)</td>
<td>n=110</td>
<td>n=18</td>
<td>n=35</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>65.3</td>
<td>77.8</td>
<td>62.9</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>34.7</td>
<td>22.2</td>
<td>37.1</td>
<td>0.53</td>
</tr>
<tr>
<td>Smoking habit (%) (n=124)</td>
<td>n=79</td>
<td>n=16</td>
<td>n=29</td>
<td></td>
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<tr>
<td>Yes</td>
<td>41.8</td>
<td>62.5</td>
<td>65.5</td>
<td>0.052</td>
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<tr>
<td>No</td>
<td>58.2</td>
<td>37.5</td>
<td>34.5</td>
<td></td>
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<tr>
<td>Familiar disease (%) (n=156)</td>
<td>n=106</td>
<td>n=16</td>
<td>n=34</td>
<td></td>
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<tr>
<td>Yes</td>
<td>17.0</td>
<td>31.3</td>
<td>5.9</td>
<td>0.07</td>
</tr>
<tr>
<td>No</td>
<td>83.0</td>
<td>68.7</td>
<td>94.1</td>
<td></td>
</tr>
<tr>
<td>ASCA (%) (n=90)</td>
<td>n=54</td>
<td>n=14</td>
<td>n=22</td>
<td></td>
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<tr>
<td>Positive</td>
<td>64.8</td>
<td>35.7</td>
<td>72.7</td>
<td>0.044</td>
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<tr>
<td>Negative</td>
<td>35.2</td>
<td>64.3</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>NOD2/CARD15 (%) (n=101)</td>
<td>n=48</td>
<td>n=18</td>
<td>n=35</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>47.9</td>
<td>44.4</td>
<td>68.6</td>
<td>0.34</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>39.6</td>
<td>38.9</td>
<td>22.8</td>
<td></td>
</tr>
<tr>
<td>Homozygotes and compound heterozygotes</td>
<td>12.5</td>
<td>16.7</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Steroid courses (No/year) (n=126)</td>
<td>n=83</td>
<td>n=15</td>
<td>n=28</td>
<td></td>
</tr>
<tr>
<td>Flares of CD (No/year) (n=126)</td>
<td>n=83</td>
<td>n=15</td>
<td>n=28</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.35 (0.48)*</td>
<td>0.23 (0.26)*</td>
<td>0.69 (0.71)*</td>
<td>0.026</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>0.74 (0.77)*</td>
<td>0.68 (0.22)*</td>
<td>1.53 (0.82)*</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Homozygotes and compound heterozygotes</td>
<td>0.25 (0.12)*</td>
<td>0.4 (0.4)*</td>
<td>0.16 (0.16)*</td>
<td>0.84</td>
</tr>
</tbody>
</table>

* B1, non-stricturing non-penetrating; B2, strictureing; B3, penetrating, according to the Vienna classification, five years after diagnosis. p values correspond to univariate analysis for each parameter using either χ² or Kruskal Wallis tests, as required.

CD, Crohn’s disease; ASCA, antischizomyces cerevisiae antibodies.

*Mean (SD).
clinically more aggressive disease. The development of penetrating lesions could be a consequence of the severity of the inflammatory reaction at the mucosal level. In contrast, stricturing disease can often be silent for a long time, manifesting only when the stricture has developed.

Active smoking was more frequent in patients who developed penetrating but also stricture disease. It was independently associated with the development of penetrating disease when patients who developed penetrating disease and patients who remained non-stricturing disease were compared. This association between smoking and penetrating disease was particularly pronounced for perianal penetrating disease. Smoking has previously been associated with more aggressive CD, but not specifically with penetrating or perianal complications. The effect of smoking could have been related to an effect on disease severity and number of flares. This association was however independent in the present study. The mechanism by which smoking may favour penetrating lesions, as well as more aggressive disease, remains to be elucidated.

As previously discussed, factors associated with intra-abdominal or perianal penetrating disease are not the same. Indeed, if a more aggressive disease (defined by the annual number of flares) seems to be associated with both subtypes of penetrating disease, it is not the case for ileal location, essentially associated with intra-abdominal penetrating disease, or for active smoking, more associated with perianal disease. These data suggest mechanisms at least partly different for the development of these two types of penetrating disease.

Furthermore, in our series, only a small minority of patients harboured both intra-abdominal and perianal penetrating disease. Therefore, they maybe represent distinct entities and should perhaps be studied independently.

When patients who developed stricturing and those who developed penetrating disease were compared, independent, independent discriminating factors were the number of flares (higher in penetrating disease, as already discussed) and ileal location, as well as the familial form of the disease (more frequent in patients who developed stricture disease). The association between stricturing disease and familial disease has already been described but was thought to be mainly linked to ileal location and early age at onset. Interestingly, a familial history of the disease was associated with stricture behavior in our study independent of ileal location or age at onset.

In the univariate analysis, ASCA were significantly more frequent in patients with penetrating lesions. An association had already been suggested between ASCA and the penetrating phenotype. In the present study however, this association was not independent, indicating that the ASCA status does not represent a significant marker associated with disease behaviour. A similar conclusion can be drawn for steroid use, associated in the univariate analysis with penetrating behaviour but no longer present after multivariate analysis. This is probably due to the correlation between the number of flares independently associated with disease behaviour and steroid use. With regard to treatment, we found no association between immunosuppressive treatment and disease behaviour. Very few patients were receiving immunosuppressive treatments however, mainly because we studied the early phase of the disease up to five years after diagnosis and because immunosuppressive treatments are often started after the development of penetrating lesions. The influence of immunosuppressive treatments or biotherapies on disease behaviour should be an area of study in a larger sample of treated patients.

Importantly, in this study we found no association between NOD2/CARD15 variants and disease behaviour. This is in contrast with reported studies. However, in those previous studies, results were partly discordant, criteria used to classify disease behaviour were inhomogeneous, and duration of disease before definition of disease behaviour was not taken into account. It must also be emphasised that the recently documented association between NOD2/CARD15 mutations and ileal location may induce secondary association with strictureing behaviour. Therefore, we believe that these previous data are inconclusive. Our study is the first to have carefully analysed this point, with a validated classification, and we can conclude that there is no major influence of NOD2/CARD15 genotype on CD behaviour. However, we cannot exclude a small influence, acting slowly and having an impact on disease behaviour after a longer period of time. This should be tested in an adequately designed study.

In conclusion, we have shown that ileal location of CD and aggressive disease with a high number of flares were independently associated with rapid development of strictureating and penetrating disease, respectively. Smoking habit was also significantly associated with the development of penetrating behaviour while there was no association between NOD2/CARD15 genotype and evolution of disease behaviour. When studying potential associations between genetic markers and disease behaviour in CD, patients should be stratified according to disease location, disease aggressiveness, and also probably smoking habit.

ACKNOWLEDGEMENTS

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Gut 2003 52: 552-557
doi: 10.1136/gut.52.4.552

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