Genetic basis for increased intestinal permeability in families with Crohn’s disease: Role of CARD15 3020insC mutation?

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Key words: intestinal barrier function, intestinal permeability, Inflammatory bowel disease, CARD15/NOD2 mutation, gene analysis

Abbreviations:
CARD15/NOD2: caspase recruitment domain family, member 15 / nucleotide-binding oligomerization domain 2
CD: Crohn’s disease
CD-R: first degree relative of a patient with CD
CD-NR: non related household member living with a patient with CD
IBD: inflammatory bowel disease
LRR: C-terminal leucine-rich repeats
NFκB: nuclear transcription factor kappa B
TNF-α: tumour necrosis factor alpha
NSAID: nonsteroidal anti-inflammatory drugs
PI: permeability index
HPLC: high performance liquid chromatography
WT: wild-type

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Abstract
A genetically impaired intestinal barrier function has long been suspected to be a predisposing factor for Crohn’s disease (CD). Recently mutations of the CARD15 gene have been identified and associated with CD. We hypothesize that a CARD15 mutation may be associated with an impaired intestinal barrier. Methods: We studied 128 patients with quiescent CD, 129 first degree relatives (CD-R), 66 non related household members (CD-NR), and 96 healthy controls. The three most common CARD15 polymorphisms (R702W, G908R, 3020insC) were analysed and the intestinal permeability was determined by the lactulose / mannitol ratio (Pl). Results: Intestinal permeability was significantly increased in CD and CD-R groups compared to CD-NR and controls. Values above the normal range were seen in 44 % of CD and 26 % of CD-R, but only in 6% of CD-NR, and in none of the controls. A household community with CD patients, representing a common environment, was not associated with increased intestinal permeability in family members. However, 40% of CD first degree relatives carrying a CARD15 3020insC mutation and 75% (3/4) of those CD-R with combined 3020insC and R702W mutations had increased intestinal permeability compared to only 15% of the wild-types, indicating a genetic influence on the barrier function. R702W and G908R mutations were not associated with a high permeability. Conclusions: In healthy first degree relatives a high mucosal permeability is associated with the presence of a CARD15 3020insC mutation. This indicates that genetic factors may be involved in the impairment of the intestinal barrier function in families with IBD.
Introduction
The integrity of the gut barrier function in patients with inflammatory bowel diseases (IBD) is known to be impaired (1-8). However, the causes and consequences of the “leaky gut” in IBD are still discussed. The leading concept assumes that the barrier dysfunction is reflecting a very early defect in the disease process that might be genetically driven and triggered by environmental factors such as luminal antigens or bacteria (9;10). By bypassing normal antigen uptake in a situation of high permeability, the induction of a physiological immune response is altered. An unsuppressed immune response is induced, initiating disease or worsening disease outcome.

To establish the role of permeability in the causality of CD, family studies have been performed. In a number of studies about 10 – 54% of first degree relatives of CD patients showed an increased intestinal permeability in absence of clinical symptoms (3;4;6;7). Although only a subgroup of relatives had increased values, data support the genetic concept. This is in line with a case report revealing changes in intestinal permeability eight years before the onset of CD (11). Interestingly, three studies showed a high prevalence of increased intestinal permeability in spouses of CD patients (7;8;12), suggesting that environmental rather than genetic factors may play a role.

Recently mutations of the CARD15/NOD2 gene have been identified and associated with CD (13-15). Up to now the relationship between IBD and the CARD15 mutation has been confirmed by many authors (16-19). The CARD15 protein is a member of the Ced-4 super family of apoptosis regulators with homology to the plant disease resistance gene products being implicated in the recognition of pathogen components (13;14). CARD15 protein acts as an intracellular receptor for the bacterial cell wall component peptidoglycan through the C-terminal leucine-rich repeats (LRRs). The bioactive component of peptidoglycan is muramyl dipeptide (20;21). This interaction leads to the activation of transcription factor NF-κB which plays a central role in innate immunity (20). The three most common genetic variants in CARD15 are the two missense mutations Arg702Trp (R702W) and Gly908Arg (G908R) and the insertion frameshift mutation at nucleotide 3020 (3020insC), the latter leading to a truncation of the C-terminal 33 amino acids in the LRR region. This frameshift mutation is associated with hyporesponsiveness to peptidoglycan and attenuated NF-κB activation (18;20). Epithelial cells transfected with the CARD15 3020insC mutant overexpressed the CARD15/NOD2 protein but displayed a diminished antibacterial defence function (22).

Keeping this in mind, the question arises whether CARD15 mutation or environmental factors are associated with the gastrointestinal barrier dysfunction. Therefore, we analysed the intestinal permeability and the three most common CARD15 polymorphisms (R702W, G908R, 3020insC) in patients with CD, their first degree relatives and non related household members. Strikingly, intestinal permeability in phenotypically normal first degree relatives of CD patients was increased compared with controls and significantly associated with the CARD15 3020insC mutation.
Methods
Participants

Patients. A total of 128 patients with confirmed diagnosis of CD were included in the present study (85 female, 43 male; age: 36 (16-63) years (median / range)). The patients were recruited from Charité University Hospital (Berlin, Germany). Diagnosis of CD was based on standard clinical, radiological, endoscopic and histological criteria. The disease was located in the terminal ileum in 37 patients (30%), the colon in 20 patients (16%), the ileocolon in 53 (40%), and the upper gastrointestinal tract in 18 patients (14%). Ninety patients were currently taking mesalamine, 31 patients oral prednisolone (n = 19 ≤ 10 mg), 19 patients oral budesonid, 5 patients sulfasalazopyridine-containing compounds, and 21 patients had no current medication. All patients were in clinical remission, the mean Crohn’s Disease Activity Index (CDAI) was 82 (range 5 – 148). None of the patients were taking antibiotics or TNF-α antibodies.

First degree relatives (CD-R). One hundred and twenty-nine first degree relatives of the patients with CD were studied (83 female, 46 male; age: 43 (15-82) years) including 62 parents, 27 siblings, and 40 children of CD patients. Forty-eight of the CD-R had been living in the same household with CD patients at least since the time of diagnosis (CD-R household members).

Non relatives (CD-NR). Sixty-six non blood relatives of the CD patients were included in the present study (23 female, 43 male; age: 38 (20-68) years). Except for one person (the stepfather of a CD patient), all CD-NR were the partners of the patients. All of them lived in the same household with the patient, the majority (n = 51) since the time of CD diagnosis.

Healthy controls. A total of 96 healthy volunteers (female 56, male 40; age: 32 (19-65) were used as controls. They were predominantly recruited in cooperation with the Department of Transfusion Medicine of Charité University Hospital.

Predetermined exclusion criteria were any (for CD-patients: concomitant) gastrointestinal or hepatobiliary disease, severe neurological, endocrine, cardiovascular, pulmonary, or renal disease, gastrectomy, colectomy, and extensive resection of the small bowel, cancer, rheumatoid based diseases, acute infections, acute urticaria and pregnancy. Alcohol and nonsteroidal anti-inflammatory drugs (NSAID) were forbidden at least 48 h before the test. CD family members (CD-R and CD-NR) and controls showing any signs or symptoms of an inflammatory bowel disease like recurrent diarrhoea, flatulence, gastrointestinal pain, and blood in the stool, or any other chronic illness were excluded from the study. Additionally, drug intake including glucocorticosteroids, antimicrobial agents, immunosuppressives, and the regular consumption of NSAIDs, e.g. for arthropathies, was not allowed for family members and controls. Persons with any previous gastrointestinal or colorectal surgery were excluded. In general, no drugs influencing gut functions including laxatives and antidiarrhoeal agents were allowed 24 hours before the onset of the test. Finally, smoking was strictly forbidden in the morning before the test and during the test. Moreover, every participant was asked to record alcohol and cigarette consumption and medicine intake for one week before the test.
**Study regime**
The intestinal permeability test was carried out at home by all persons. Blood samples for genetic analysis were taken in a subgroup of study participants, either at the outpatient department of the clinical centres involved or at their family doctors. A standardized questionnaire was used for data inquiry.

**Intestinal permeability**
The intestinal permeability was assessed using a sugar-drink-test as previously described in detail (23). The test is based in principle on the measurement of the urinary excretion of orally administered non-metabolised sugar probe molecules; the lactulose / mannitol ratio (permeability index, PI) served as marker for intestinal permeability.

After an overnight fast, each subject provided a pre-test urine sample. Then they drank a solution containing 10 g lactulose, and 5 g mannitol dissolved in 100 ml water. Urine was collected over 5 h with sodium azide as preservative. Subjects went without food during the test but were allowed to drink water after 2 h. Total urine volume was recorded on completion of the test and a 10 ml aliquot was stored at minus 20°C until analysis.

For sample preparation the protein was removed with sulfosalicylic acid and the urine was desalted with Amberlite MB-3 resin in the acetate form. Using meso-erythritol and turanose as internal standards the sugars were separated, analysed and quantified by HPLC with pulsed electrochemical detection (Dionex, Idstein, Germany); chromatography module: 250 x 40 mm Carbopac PA-1 column (Dionex); eluent 150 mmol NaOH; flow; 1ml/min. Results were expressed as the percentage recovery of the ingested dose of the sugars.

**Gene analysis**
All methods used for gene analysis were described previously in detail (19). Briefly, genomic DNA was prepared from peripheral blood using commercially available extraction columns (QIAmp Blood Kit, QIAGEN, Hilden, Germany). After amplification of the Exon 11 of the CARD15 gene and cycle sequencing using an ABI 310 automatic sequencer (Applied Biosystems, Weiterstadt, Germany), the genotyping of the three CARD15 polymorphisms (R702W, G908R, 3020insC) were performed using the fluorogenic 5´-nuclease assay. Primers and probes were the same as previously described (19).

**Statistics**
Differences in permeability data between the study groups were analysed using the Kruskal-Wallis test and, in case of significance, using the Mann-Whitney-U test. The level of significance (α) was chosen at 5%. An α-adjustment was applied by Bonferroni procedure. Differences between incidences were tested by cross tables and Chi-square statistics.

The upper limit of normal intestinal permeability was defined as mean value + 2SD of the control group (= 0.03) as previously described (5;23).
Ethical considerations
The study was approved by the ethics commission of the Charité University Hospital and informed consent was obtained from each participant.

Results
Intestinal permeability
The intestinal permeability was significantly increased in CD patients and in CD-R compared to controls and CD-NR (Figure 1). Values above the normal range were seen in 44% of CD patients and 26% of CD-R, but only in 6% of CD-NR, and in none of the controls. This already speaks against the role of an environmental factor being predominantly responsible for the barrier dysfunction. To further differentiate between genetic and environmental factors we concentrated on the group of CD first degree relatives and used the household community with the CD patients to investigate the influence of environmental factors on the intestinal permeability. Thirty-seven percent (n = 48) of CD-R lived in the same household with the patient since the time of CD diagnosis (CD-R household members). Forty-seven percent (n = 61) of CD-R lived in a different household than the patient, both at the time of CD diagnosis and at the time of the test (CD-R non-household members). As seen in Table 1 the intestinal permeability did not differ between these two groups, indicating that household community, as an environmental factor, does not explain the general differences in intestinal barrier function described above.

We further analysed the effect of smoking habits in each group. The portion of smokers showing a PI above the normal limit was higher compared to non-smokers (61% vs. 37%; p=0.019, Fisher’s exact) in CD patients, but in none of the other groups (CD-R p=0.602; CD-NR p=0.563). The median values of the intestinal permeability did not differ between smokers and non-smokers in any of the groups. Additionally, a slight gender disproportion in the CD-R and CD-NR groups had no significant effect on the intestinal permeability values (data not shown).

Table 1: Intestinal permeability in CD-R subgroups

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>PI (lac % / man %)</th>
<th>Persons with high permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (Interquartile range)</td>
<td></td>
</tr>
<tr>
<td>CD-R (household members)</td>
<td>48</td>
<td>0.019 (0.015 - 0.030)</td>
<td>25 % (12)</td>
</tr>
<tr>
<td>CD-R (non household members)</td>
<td>61</td>
<td>0.020 (0.017 - 0.034)</td>
<td>31 % (19)</td>
</tr>
</tbody>
</table>

The intestinal permeability did not differ between CD-R subgroups. CD-R household members were living in the same household with the patients at the time of diagnosis and at the time of the test; CD-R non household members were living on their own during the whole period; Interquartile range: 25th - 75th percentile.
Genetic analysis

Three major polymorphisms within the coding region of the **CARD15** gene (3020insC, R702W, G908R) have been associated with CD. The results of the present study are illustrated in Table 2. A significantly higher prevalence of the frameshift mutation 3020insC was found in CD patients and their first degree relatives as compared to controls. The portion of the heterozygous plus homozygous mutations was 22%, being similar to that found in the CD-R group (23%). In the CD-NR group the corresponding value was 12%. A similar tendency was obvious for the R702W mutation, while data for the G908R did not differ between the study groups (Table 2).

Table 2: Prevalence of **CARD15** mutations

Overall: number of persons carrying at least one mutation; p values for differences in frequency

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>CD</th>
<th>CD-R</th>
<th>CD-NR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3020insC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild-type</td>
<td>98 % (61)</td>
<td>78 % (94)</td>
<td>77 % (82)</td>
<td>88 % (44)</td>
</tr>
<tr>
<td>heterozygous mutant</td>
<td>2 % (1)</td>
<td>18 % (22)</td>
<td>22 % (23)</td>
<td>10 % (5)</td>
</tr>
<tr>
<td>homozygous mutant</td>
<td>4 % (5)</td>
<td>1 % (1)</td>
<td>2 % (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0005</td>
<td>p &lt; 0.0005</td>
<td>p = 0.044</td>
<td></td>
</tr>
<tr>
<td><strong>R702W</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild-type</td>
<td>94 % (58)</td>
<td>83 % (100)</td>
<td>84 % (89)</td>
<td>88 % (44)</td>
</tr>
<tr>
<td>heterozygous mutant</td>
<td>6 % (4)</td>
<td>14 % (17)</td>
<td>15 % (16)</td>
<td>12 % (6)</td>
</tr>
<tr>
<td>homozygous mutant</td>
<td>3 % (3)</td>
<td>1 % (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.06</td>
<td>p = 0.091</td>
<td></td>
<td>p = 0.337</td>
</tr>
<tr>
<td><strong>G908R</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild-type</td>
<td>97 % (60)</td>
<td>92 % (110)</td>
<td>98 % (103)</td>
<td>98 % (49)</td>
</tr>
<tr>
<td>heterozygous mutant</td>
<td>3 % (2)</td>
<td>8 % (10)</td>
<td>2 % (2)</td>
<td>2 % (1)</td>
</tr>
<tr>
<td></td>
<td>p = 0.343</td>
<td>p = 0.628</td>
<td></td>
<td>p = 1.000</td>
</tr>
</tbody>
</table>

**Overall**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CD</th>
<th>CD-R</th>
<th>CD-NR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11 % (7)</td>
<td>43 % (52)</td>
<td>37 % (39)</td>
<td>24 % (12)</td>
</tr>
</tbody>
</table>

Association between mucosal permeability and **CARD15** mutations

Looking for a possible link between the prevalence of the **CARD15** mutation and the intestinal barrier function, the individual study groups were divided into subgroups according to the occurrence of a mutation (Table 3). CD patients without (wild-types) and with at least one **CARD15** mutation (mutants) did not differ in respect to their mucosal permeability. In CD wild-types and patients with 3020insC mutation the frequencies of elevated permeability were 44% and 52%, respectively. In contrast, in healthy CD first degree relatives we found a significant association between **CARD15** 3020insC mutation and intestinal permeability (Table 3). Only 15% of the wild-types, but 40% of 3020insC mutation carriers had increased values. In addition, the median values of intestinal permeability were significantly
higher in the CD-R with 3020insC mutation compared to the CD-R wild-types (Figure 2). In four CD-R combined mutations (3020insC and R702W) occurred; three of them also had an increased permeability. In contrast neither R702W nor G908R alone showed a clear association. In CD-NR all of the mutation carriers had a normal permeability (Table 3).
Table 3: Frequencies of normal and high intestinal permeability in respect to the occurrence of CARD15 mutations

<table>
<thead>
<tr>
<th>PI</th>
<th>WT</th>
<th>3020insC</th>
<th>R702W</th>
<th>G908R</th>
<th>3020insC and R702W</th>
<th>3020insC and G908R</th>
<th>R702W and G908R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 68</td>
<td>n = 23</td>
<td>n = 16</td>
<td>n = 8</td>
<td>n = 3</td>
<td>n = 1</td>
<td>n = 1</td>
</tr>
<tr>
<td>CD</td>
<td>normal</td>
<td>38 (56%)</td>
<td>11 (48%)</td>
<td>8 (50%)</td>
<td>5 (63%)</td>
<td>2 (67%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>30 (44%)</td>
<td>12 (52%)</td>
<td>8 (50%)</td>
<td>3 (37%)</td>
<td>1 (33%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p = 0.629</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|        | n = 66    | n = 20   | n = 13| n = 2 | n = 4               | -                   | -                 |
| CD-R   | normal    | 56 (85%) | 12 (60%)*| 10 (77%)| 2 (100%)        | 1 (25%)            |                   |
|        | high      | 10 (15%) | 8 (40%)*| 3 (23%)| -                   | 3 (75%)            |                   |
|        |           |          |       |       | p = 0.027          |                     |                   |

|        | n = 38    | n = 6    | n = 5 | -    | -                   | -                   | n = 1             |
| CD-NR  | normal    | 36 (95%) | 6 (100%)| 5 (100%)| -                   | -                   | 1 (100%)          |
|        | high      | 2 (5%)   | -     | -    | -                   | -                   |                   |
|        |           |          |       | p = 1.000 |                     |                     |                   |

WT: Wild-type (without CARD15 mutation) 3020 insC, R702W, and G908R mutations of the CARD15 gene (homozygous plus heterozygous); * significantly different incidence of high and normal permeability between wild-type and mutation subgroups. Normal permeability: PI ≤ 0.03, high permeability: PI > 0.03; chi-square test paired comparison (Fisher’s exact).
Discussion

The present study revealed three main findings: First: Healthy first degree relatives of patients with CD showed increased intestinal permeability in contrast to unrelated household members and in contrast to controls. Second: The prevalence of the CARD15 3020insC mutation was similar in first degree relatives and CD patients and higher compared to controls. Third: In healthy first degree relatives a high mucosal permeability and the presence of a CARD15 3020insC mutation were significantly associated.

Based on a particularly large sample size compared to others (1;4;6-8) our analysis clearly reveals intestinal permeability defects in 26 % (34/129) of healthy first degree CD relatives. Furthermore, there was no significant difference between those living in the same household with a CD patient since the time of diagnosis and those living on their own. Thus, the parameter “sharing an environment” does obviously not explain the different permeability patterns in CD first degree relatives. The changes in permeability seem to reflect genetic rather than environmental factors. This is in line with the results in the group of non blood relatives. All of them shared a household with a patient, but only 6 % (4/66) had a PI above the normal level. However, data in the literature appear contradictory. Some studies not only documented a high baseline permeability in related (3;4;6-8) but also in unrelated household members (spouses) of CD patients (7;8;12). These reports were partly limited by sample size and some used a lower “upper limit of normality” for the permeability values than in this study (e.g.: 0.0195 (8) and 0.0170 (7)), which might have a slight effect on the frequency distribution of the data. However, under close scrutiny, they also revealed clear evidence that epithelial hyperpermeability of the intestine, in response to insults causing barrier dysfunction, was restricted to the CD first degree relatives and was not seen in spouses (8). Summing up, data are pointing towards a genetic rather than an environmental background of the intestinal barrier dysfunction in CD. Our results add further support to the role and the value of the intestinal barrier function as a subclinical marker in families with CD. The question, whether the increased intestinal permeability may be a primary or a causative factor in the disease development, cannot be answered from the present data. It is assumed by some authors that a slight inflammation may be a primary event preceding the barrier disruption. A recent study using faecal calprotectin concentration for detection of intestinal inflammation reported 88% (n=43) of CD patients, 49% (n=74) of CD first degree relatives, and 13% (n=2) of CD spouses having abnormally high values (24). The authors proposed a genetic basis for a subclinical inflammation representing a risk factor for CD. Others reported a relationship between faecal calprotectin and intestinal permeability (25). It could well be that a subliminal inflammation may precede the barrier disruption. However, a definition of causality is difficult in this case because both factors represent at the same time also secondary factors, perpetuating and worsening the processes in the intestinal mucosa. In our opinion neither method (faecal calprotection detection nor in the vivo permeability measurement) provides sufficient sensitivity to answer this question. The analysis of mucosal tight junction components and metabolisms in CD first degree relatives, for example, may be more useful in this matter. However, the results of Thjodleifsson et al. 2003 (24) stress the importance of a genetic basis of CD and support the role of subclinical markers.
A possible genetic mechanism involved in barrier dysfunction in healthy family members is unknown. An interesting candidate for such a genetic factor is the *CARD15* gene or rather its mutations associated with CD. In accordance with the main findings in European, Canadian, and US Caucasian populations (14-18;26), our gene analysis reveals a significantly higher prevalence of the *CARD15* 3020insC mutation in CD patients compared to controls. Interestingly, this was also true for the CD first degree relatives. Values were similar to those of the patients and, in contrast to the non blood relatives, significantly higher compared to controls. Obviously, there was a transmission of the polymorphisms within genetically related persons which supports very recent data (27). However, the striking finding of the present study was that the healthy first degree relatives carrying a 3020insC mutation were more often characterized by high intestinal permeability than the wild-types. Eighty-five percent of the CD-R wild-types had normal permeability.

A relationship between CD and the *CARD15* mutation is assumed. However, *CARD15* mutations are not generally present in all CD patients, and they are completely uncommon in some populations, for example they were not found in the Japanese, or Chinese Han populations (28;29) or an Icelandic population as seen in the study of Thjodleifsson et al. 2003 (24). Thus *CARD15* mutation may be one risk factor for CD development, but obviously, it is not the sole factor. Interestingly, we and others found that the 3020insC is associated with a special phenotype of CD with younger age at diagnosis, ileal involvement, ileocaecal resections, and a high risk of postoperative relapse and re-operation (16;17;19). 3020insC might therefore characterise a subgroup or subtype of CD associated mainly with ileitis. Regarding a possible link to the intestinal barrier function, the physiological consequences of a *CARD15* mutation are crucial. As already mentioned the *CARD15/NOD2* protein is an intracellular receptor for muramyl dipeptide, a conserved structure in bacterial peptidoglycan (20;21). In human intestinal tissue the protein is expressed predominantly in Paneth cells in the crypts of the terminal ileum (30). Clear functional consequences have been demonstrated mainly for the frameshift mutation 3020insC. 3020insC results in a diminished bacterial recognition and hyporesponsiveness to bacterial muramyl dipeptide showing attenuated or complete loss of NF-κB activation. A signaling defect of the innate immunity was indicated (18;20;22;31). Studies in mice (31) and humans (32) reported a 3020insC associated, diminished expression of antimicrobial peptides such as defensins in the intestinal Paneth cells. The authors speculated that defensin deficiency may lead to an impaired mucosal barrier and to susceptibility to bacterial invasion which could trigger inflammation and loss of tolerance against the luminal flora (32). A direct effect of bacterial products on the tight junction integrity (33) or a secondarily exaggerated local inflammatory response of the adaptive immune system, i.e. high TNF-α or NF-κB production (13), may cause or facilitate a barrier break. Both of these pathways are possible and might play a role. They might gain special importance representing an early event probably pointing towards a risk in a subgroup of persons. Interestingly, an older study reported an abnormal faecal flora in patients with CD and their first degree relatives (34).
However, if there is an association of the CARD15 mutation and the impaired barrier function, why is this association not obvious in CD patients but only in a subgroup of healthy first degree relatives of CD patients? We are facing two completely different situations. One is the healthy person being a first degree relative of a CD patient. This person has no clinical symptoms of CD but has a CARD15 mutation and an impaired barrier function which may bear a risk but which may not develop any clinical relevance. The other situation is the confirmed diagnosis of a chronic inflammatory bowel disease, i.e. CD. In our study 44% of CD patients without any CARD15 mutation, i.e. wild-types, showed pathologically high permeability values. In current CD various factors, including the dynamism of the inflamed, disturbed barrier itself, influence the barrier function. Early events could be easily masked. These factors are mainly drugs such as prednisolone or mesalamine (35), or effects of mal- and dysnutrition (36;37), stress (38;39) or changes in luminal bacterial load and composition (34;40;41), which are all associated with CD and which are accompanied by reinforcement or impairment of the intestinal barrier.

Summing up, the present data are pointing towards a genetic rather than an environmental basis of the intestinal barrier dysfunction in CD. Data allow us to speculate that CARD15 3020insC mutation could be one genetic factor involved in the impairment of the intestinal barrier function. However, it is obvious that this is not the only factor. The association of CARD15 gene mutation and intestinal hyperpermeability in healthy first degree CD relatives may be one step in the identification of other target genes involved in similar processes or probably interacting with the CARD15 gene as recently discussed (42;43) and proposed for the DLR5 gene (44). Our data point towards a very early step in the disease process. Considering the high intestinal bacterial load in CD patients compared to controls (41) an early barrier dysfunction gains special significance in respect to the pathogenesis of CD. However, longitudinal studies in CD families are necessary to investigate which additional factors probably lead to the outbreak of the disease.
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Competing interest statement
On behalf of all authors the corresponding author states that non of the authors has to declare a competing interest.
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Figure legends

**Figure 1:** Intestinal permeability in healthy controls, in patients with Crohn’s disease (CD), their first degree relatives (CD-R) and non blood relatives (CD-NR). The intestinal permeability was increased in CD and CD-R compared to controls.

**Figure 2:** Intestinal permeability in Crohn’s disease first degree relatives without a *CARD15* mutation (CD-R WT) and with 3020insC or 3020insC-R702W mutations (CD-R 3020insC). The intestinal permeability was significantly increased in CD-R with mutation compared to CD-R without mutation.
Intestinal permeability in first degree relatives
Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation?

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