Familial Aggregation and Antimicrobial Response Dose-Dependently Affect the Risk for Crohn's Disease

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Background: An increased risk of Crohn's disease (CD) has been reported consistently in first-degree relatives of patients. Our aim was to test whether a combination of CD-associated genes involved in innate immunity and/or antibody responses to microbial antigens may be valuable in identifying healthy relatives at risk.

Methods: We investigated 86 families from Belgium and northern France, 45 with at least 3 first-degree relatives with CD, 24 with a single case, and 17 control families without inflammatory bowel disease (IBD). The cohort consisted of 186 CD patients, 290 healthy relatives, and 142 controls (total 618). Genetic (*NOD2*, *NOD1*, *TLR4*, *CARD8*) and serologic markers (ASCA, ACMA, ALCA, ACCA, AΣMA, OmpC, CBir1, I2) were determined in all subjects. All Belgian families were prospectively followed up for 54 months.

Results: In multiple-affected families, an increment of affected first-degree relatives and of positive antibodies were additive risks factors for CD ($P < 0.0001$), independent of *NOD2* mutations. When comparing subjects from multiple-affected families, having 3 additional first-degree relatives with CD and 1 additional positive antibody increased the odds for CD to 9.19 (95% confidence interval [CI]: 4.07–20.80). After a follow-up of 54 months among all Belgian families, a total of 4 new diagnoses of IBD were confirmed in the multiple-affected families only, resulting in a 57-fold increase in incidence within multiple-affected families compared to the known incidence of IBD in our region.

Conclusions: We found an additive risk increment for CD in subjects from multicase families per additional affected relative and per additional positive antibody, independent of *NOD2*. Furthermore, a very high disease incidence was observed in these multiple-affected families.

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Key Words: antimicrobial antibodies, disease prediction, familial aggregation, IBD incidence, *NOD2*

Crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic relapsing inflammation, resulting from a complex interplay between environmental risk factors and immunological changes in a genetically susceptible host. Having a relative with either CD or UC is a consistent risk factor to either of these inflammatory bowel diseases (IBDs). Overall, between 2% and 22% of CD patients report at least 1 first-degree relative also affected with IBD.[1] Among siblings, the risk is greater as compared to other familial relations.[2–8] An exceptionally high frequency of familial CD has been reported in northern France and Belgium.[9] We previously performed a study of environmental risk factors in Belgian families with at least 3 members affected with CD. Among the risk factors were childhood diseases and consumption of unpasteurized milk and uncooked pork, whereas protective factors included contact with pets, the consumption of oats, and the consumption of tap water compared to well water during childhood.[10]

Since having a first-degree relative with CD is an important risk factor, one would expect that in families with several affected first-degree members the unaffected relatives are at much higher risk for CD development than the general population. In several studies, children with both parents affected with CD carried a risk as high as 36%.[11–13] Attempts to distinguish sporadic from familial CD patients have been undertaken.[14,15] Patients with a familial history

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of CD have been shown to have an earlier age at onset and it has been hypothesized that this was due to genetic anticipation.[16,17] As clustering of risk factors is expected in families with multiple affected subjects, we hypothesized that unaffected relatives may also carry more risk factors. However, it is unclear if overall they carry more risk factors or specific ones and, if so, which and how many risk factors are necessary and/or sufficient for IBD development.

Both genetic and environmental factors are shared within families. Multiple-case families are most likely to carry a strong genetic predisposition[18] and several genetic factors have been associated with IBD so far. Notably, the CD-associated *NOD2* mutation is thought to be associated with familial history.[19] However, the low penetrance limits the clinical value of *NOD2* testing and encourages combining several genetic susceptibility factors. Environmental factors are also involved in disease pathogenesis. In this respect, several antibodies to bacterial and fungal epitopes have been associated with the disease.[20] Although most of these serologic markers have solely a modest accuracy in detecting CD, by combining them up to 79.9% of CD patients have positive antibody responses to microbial antigens.[21,22] More recently, antibodies have been proposed for disease prediction[23] and stratification.[24–26] As disease phenotypes of CD are known to also have a high concordance within families,[5,27] it is likely that antibodies may cluster within families.

In this study we aimed to assess the accuracy of genetic and environmental risk factors in predicting disease incidence by comparing patients and their unaffected relatives from multicase families with sporadic and control families. We therefore prospectively followed up the health status of controls and the unaffected relatives and spouses of well-characterized CD patients.

TABLE 1. Description of the Total Study Cohort

	Multiple Affected Families (>3 CD)	Families with 1 CD Patient	Control Families
Total number of families (total number of CD patients)	45 (162)	24 (24)	17 (0)
Belgian families	21	4	10
French families	24	20	7
Mean number of relatives per family	5	5	5
Females (%)	53	51	53
Mean age (years)	51	49	51

TABLE 2. Description of the Multicase Families

Disease Status 2000	CD Patients ($n = 162$)		Unaffected Related Controls ($n = 219$)		Total
Nationality/sex	Male	Female	Male	Female	
Belgian	32	41	37	46	156
French	39	50	66	70	225
Total	71	91	103	116	381

MATERIALS AND METHODS

Study Cohort

We studied a total of 86 families from Belgium and northern France of whom 45 were multiple affected families with at least 3 first-degree relatives with CD, 24 families were sporadic (i.e., only 1 affected patient), and 17 were healthy control families (Tables 1, 2). The sporadic families and the control families were recruited per country and they belonged to the same generation, had an equal composition of males and females, and consisted of a comparable number of persons within the family. In 2 families with multiple CD patients there was also 1 UC patient.

All participants gave blood after written informed consent for serologic and genetic testing. Medical records of all affected members of the families were reviewed by independent gastroenterologists from 2 different university hospitals. The surgery, histology, and clinical records were studied. The study was approved by the Ethics Committee of the Catholic University of Leuven and by the CCPPRB of Lille (ref. CP 00/60).

To assess our specific aims, 6 groups of interest were identified and are listed in Table 3. We first compared patient

TABLE 3. Six Groups of Interest Within the Total Cohort

No.	Category	Number of Males	Number of Females	Total
1	CD patients from multi-case families	71	91	162
2	CD patients from sporadic families	7	17	24
3	Unaffected from multi-case families	103	116	219
4	Unaffected from sporadic families	36	35	71
5	Spouses of IBD patients	20	17	37
6	Pure controls	48	57	105
	Total	285	333	618

groups including patients from multicase families (group 1) with the sporadic patients (group 2). Second, we compared the groups of the unaffected relatives from multicase families (group 3) and from sporadic case families (group 4), the group of spouses of IBD patients (group 5), and the healthy control group (group 6). In group 5 only spouses who lived together with an affected subject but who did not have any genetic link with an IBD patient were studied.

Markers Assessed as Risk Factors

NOD1 (rs2075822 and rs2907748), *CARD8* (rs2043211), *NOD2* (rs2066844, rs2066845, and rs2066847), and *TLR4* (rs4986790) were studied using polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP). To allow a rapid genotyping of the insertion-deletion polymorphism of *NOD1* (rs6958571), a nonisotopic PCR/single-strand conformation polymorphism (SSCP) strategy was developed (details available upon request).

Serological titers of anti-*Saccharomyces cerevisiae* antibody (ASCA), anti-*Candida albicans* mannan antibody[28] (ACMA), anti-chitobioside carbohydrate and anti-laminaribioside carbohydrate antibodies (ACCA and ALCA, respectively), anti-synthetic mannoside antibodies[29] (AΣMA), anti-outer membrane porin C antibody (OmpC), anti-bacterial sequence I2 antibody (anti-I2), and antibacterial flagellin antibody (CBir1) were determined using specific enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer's guidelines (details available upon request).

Statistical Analyses

The statistical analyses were performed on the whole cohort, excluding the new cases that arose since 2000 and the 2 UC patients. Hence, a total of 618 persons were used, which included 383 French and 235 Belgian subjects. To correct for ascertainment bias, the analyses were done with and without the probands.[30] We used the newly identified cases of IBD within the cohort to calculate an incidence rate over the follow-up interval.

To compensate for unobserved data (for instance, average per marker missing rate about 17%) an imputation technique was applied. For the genetic imputations we used Merlin-1.1.2,[31] which exploits information from flanking markers and family structure during the imputation process. When genotypes are missing, genotype probabilities are outputted per subject and per gene. Genotypes with probabilities lower than 0.95 were used in R 2.7.0 software to randomly generated genotypes based on the probabilities from Merlin-1.1.2. In pedigrees with missing genotypes in different generations, first the imputations of the founders were done based on the known genotypes of offspring, and in a next step the missing genotypes in the offspring were calculated.

For the imputation of missing serological data a more intuitive method was used. In subjects with a missing value for a serological marker the average of this marker in comparable subjects in the cohort (based on the categories in Table 3) was used as an estimate for the missing value. After the subject-level imputation step, individual's sum scores were derived. Hence, imputations were performed before deriving a summary serological measure per individual. By using these summary measures in further analyses instead of individual serological markers, we reduced potential bias introduced by the aforementioned mean imputation procedure.

In terms of an association analysis, as genotype frequencies between diseased and nondiseased persons could not be evaluated by standard χ^2 tests because of the presence of familial relationships, the FBAT program (Family-Based Association Tests, v. 1.5.5) was used to perform both biallelic and multiallelic tests of genetic association. To account for nonindependence between nuclear families, empirical variances were applied to the FBAT statistics. Quantitative traits adjusted for covariates were further analyzed by using the Windows executable file pbat32.exe.[32] An exploratory genetic interaction analysis was performed using the software MDR-PDT, which is particularly designed for epistasis detection with family-based designs.[33]

To further investigate the nature of familial clustering, we used PROC GLM module of the SAS v. 9 software (Cary, NC) to analyze both genetic and seroreactivity differences between the different subgroups in our cohort, accounting for unbalanced data (i.e., taking unequal numbers in the groups into account). In addition, the Statistical Analysis for Genetic Epidemiology (S.A.G.E.) software was used[34] to evaluate clustering of CD status and serologic factors within the families.

As missing data handling strategies may introduce bias, in particular when the missing data process is not "missing completely at random" (MCAR),[35] all results were subjected to a sensitivity analysis.

Risk factors for CD in multiple affected families were identified with PROC GENMOD of the SAS v. 9 software, thereby accounting for family correlatedness.

RESULTS

Association Analyses for CD

The distribution of CD patients and controls did not differ significantly between the Belgian and the French subgroups ($P = 0.83$). Nationality, however, was included as a confounding factor in all analyses for its potential to act as an effect modifier. All known associations between the studied markers and CD status could be confirmed in this cohort using FBAT statistics that takes the family

TABLE 4. Risk Factors for CD in Multicase Families, Calculated Without Probands

Risk Factor	Odds Ratio for CD	95% Confidence Interval
Increment of one first-degree relative with CD	1.53	1.27–1.84
Increment of two first-degree relatives with CD	2.33	1.62–3.37
Increment of three first-degree relatives with CD	3.57	2.06–6.19
One additional antibody tested positive	2.58	1.98–3.36
Two additional antibodies tested positive	6.65	3.91–11.30
Three additional antibodies tested positive	17.14	7.73–38.01

structure into account (data not shown). When using PROC GENMOD with general correlation structure between family members, several factors showed an association with disease status. Those associations were consistent with the previous literature and consistent between the Belgian and the French cohorts (Suppl. Table 1).

When assessing risk factors for CD in the multiplex families, a cumulative effect of number of first-degree affected relatives and number of positive antibodies was detected ($P < 0.0001$), even when the probands were excluded from the analyses (Table 4). For a healthy individual with 1 first-degree relative with CD, the odds ratio for CD development was 1.53 (95% confidence interval [CI]: 1.27–1.84) and increased to 2.33 (95% CI: 1.62–3.37) and 3.57 (95% CI: 2.06–6.19) if he/she had 2 or 3 affected first-degree relatives, respectively. A similar dose increase in risk was observed for the serological response and this odds ratio appeared even stronger than the risk associated with having an affected relative (Table 4). Notably, the risk increased from 2.58 (95% CI: 1.98–3.36) to 17.14 (95% CI: 7.73–38.01) with 3 instead of 1 additional positive antibody. Importantly, since there was no interaction between the increase in risk from having an affected relative and from carrying positive antibodies, these effects were cumulative. When comparing subjects from multiple affected families, having 3 additional first-degree relatives with CD and 1 additional positive antibody increased the odds for CD to 9.19 (95% CI: 4.07–20.80). We also distinguished between subjects from multicase families with and without *NOD2* mutations and the results are presented in Table 5.

Since there was a significant interaction between the number of first-degree relatives affected with CD and the number of detected mutations, no odds could be calculated based on mutations in the multiple affected families.

Interaction analyses using MDR-PDT did not highlight significant clusters of genetic markers in association with CD.

Clustering of Risk Factors Within Families

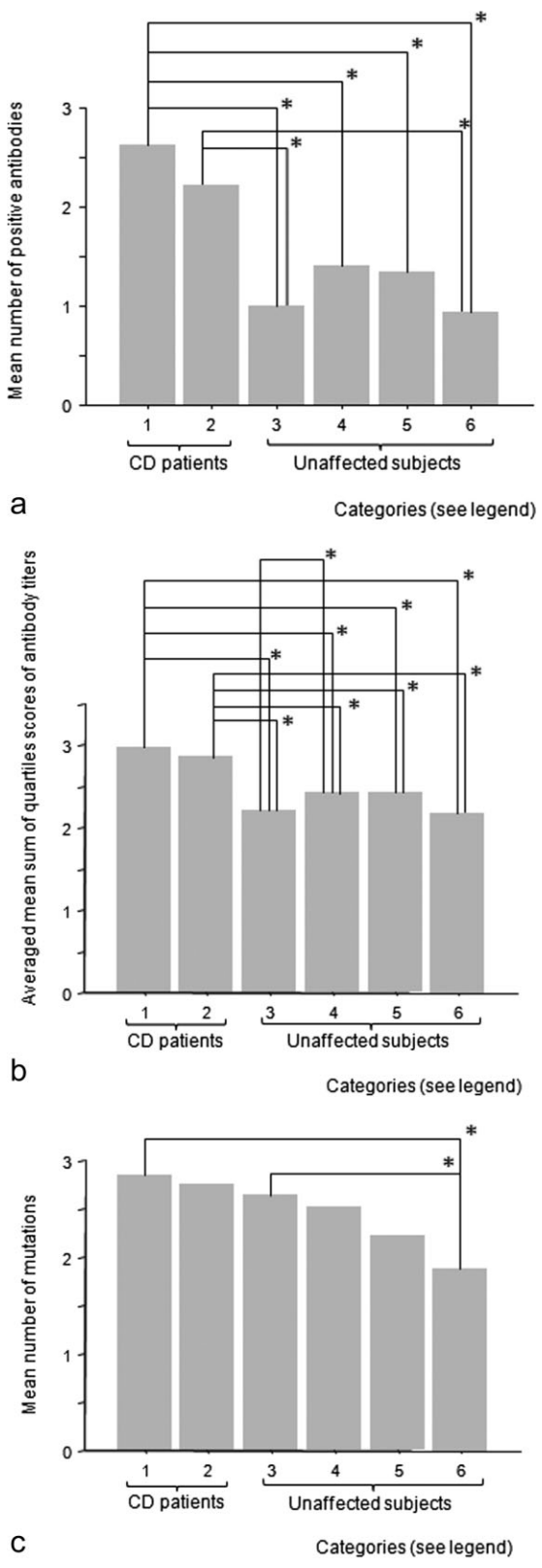
Concordance rates for disease were investigated as well as correlation rates with serological markers in the entire cohort for several multigenerational family pairs; these were in line with expectations (results not shown).

Visual inspection of the number of antibodies and genetic mutations in each pedigree showed no evidence for clustering of markers within pedigrees. The number of positive antibodies and mutations were analyzed in relation to the affection status and relationship within our cohort. Significant differences between the categories described in Table 3 were found and are shown in Figure 1a–c ($P < 0.0001$). Using pairwise comparisons, patients clearly had more positive antibodies compared to unaffected subjects. For the unaffected group, there was no difference between relatives and controls or spouses.

The averaged sum of quartile scores for the antibody titers were compared and are shown in Figure 1b. The profile of the graph is similar to that of Figure 1a. All pairwise

TABLE 5. Risk Factors for CD in Multicase Families, Calculated Without Probands, Subdivided Based on *NOD2* Mutations

Subjects Without *NOD2* Mutations	Odds Ratio for CD	95% Confidence Interval
Increment of one first-degree relative with CD	1.92	1.38–2.68
One additional antibody tested positive	2.19	1.52–3.14
Subjects with *NOD2* mutations		
Increment of one first-degree relative with CD	1.32	1.13–1.55
One additional antibody tested positive	3.00	2.05–4.39



comparisons of the intensities of antibody reactions between patients and unaffected subjects were significantly different. In the group of unaffected relatives the average intensities of the antibodies in the relatives of sporadic patients was significantly higher than those of relatives of familial patients, as indicated in Figure 1b with an asterisk.

For the mean number of mutations (Fig. 1c) the differences between the categories were also highly significant. Overall, patients again have a higher average number of mutations but in pairwise comparison only the higher number of mutations in patients from multicase families and their unaffected relatives compared with healthy controls was significantly different, as shown in Figure 1c.

Differences between the categories were also assessed per genetic marker. These differences were significant for *NOD2* and for *TLR4* (Fig. 2a, $P < 0.0001$, and Fig. 2b, $P = 0.0085$). Subjects from multicase families clearly had more *NOD2* mutations compared pairwise to unaffected subjects. The number of *NOD2* mutations of patients from multicase families was also pairwise significantly higher than the number of *NOD2* mutations from sporadic cases. The pairwise difference in *TLR4* mutations was only significant for the patients from multicase families versus pure controls after correction for multiple testing, however.

In the multicase families we also assessed the number of markers in relation to the number of first-degree relatives with CD. Instead of categorizing the subjects based on the type of family they belonged to, all unaffected relatives were ordered according to their respective number of first-degree relatives affected with CD. A higher number of mutations was found with an increasing number of first-degree relatives with CD (Fig. 3). This association was significant ($P = 0.0195$). No significant relation between the number of antibodies and the number of first-degree relatives could be found, however.

Sensitivity Analyses for Missing Data Strategies

To assess how sensitive the results may be to our missing data handling, we computed the number of FBAT informative families (Table 6) before and after Merlin imputations of genotypes. Obviously the number of

FIGURE 1. a: Mean number of positive antibodies per category. b: Averaged mean sum of quartile scores of antibody titers per category. c: Mean number of mutations per category. 1, CD patients from multicase families ($n = 162$); 2, CD patients from single-case families ($n = 24$); 3, unaffected relatives from multicase families ($n = 219$); 4, unaffected relatives from single-case families ($n = 71$); 5, spouses of IBD patients ($n = 37$); 6, pure controls ($n = 105$). *Indicates additional significance at 0.05 level for pairwise comparison after correction for multiple testing.

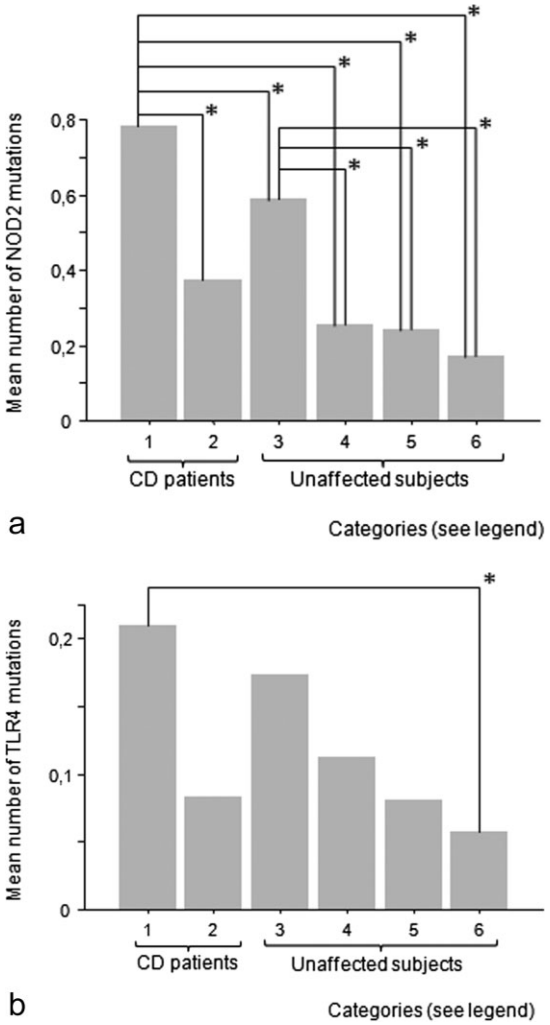


FIGURE 2. a: Mean number of *NOD2* mutations per category. b: Mean number of *TLR4* mutations per category. 1, CD patients from multicase families ($n = 162$); 2, CD patients from single-case families ($n = 24$); 3, unaffected relatives from multicase families ($n = 219$); 4, unaffected relatives from single-case families ($n = 71$); 5, spouses of IBD patients ($n = 37$); 6, pure controls ($n = 105$). *Indicates additional significance at 0.05 level for pairwise comparison after correction for multiple testing.

informative families increases when more data become available. Despite this fact, all FBAT analyses before and after imputation were comparable in the sense that (un)significant results remained (un)significant. Moreover, the direction of association effects remained constant. This was not entirely surprising since about 42% of the imputations were done with an accuracy probability of 95% or more. The imputation procedure did not significantly change allele frequency estimates (see Table 6; paired *t*-test *P*-value: 1.000). This led us to believe in the validity of the implemented missing data strategy.

Prospective Follow-up of Families

All Belgian families returned our follow-up survey. Four new diagnoses of IBD (2 CD and 1 UC in male first-degree relatives and 1 CD in a fourth-degree female) were made from 2000 to 2005 in 4 different multiple affected families compared to none in either sporadic or healthy control families. The characteristics of the newly diagnosed first-degree relatives are displayed in Table 7. None of the Belgian families where new first-degree cases were reported after prospective follow-up had a high genetic ($\geq$3 mutations on average) or a high serologic ($\geq$2 antibodies on average) load. The family where CD was discovered in a fourth-degree relative, however, had a very high genetic load (mean number of mutations 5.14).

All newly diagnosed first-degree relatives were male. Their age at diagnosis varied between 23 and 44 years. Among the newly diagnosed CD patients, patient 1 carried a *NOD2* variant (heterozygote for rs2066844 mutation) as well as *NOD1* mutations (heterozygote for rs2075822, rs2907748, and rs6958571). CD patient 1 also expressed positive ASCA in combination with CBir1 antibodies. The other first-degree CD patient (patient 2) and the newly diagnosed first-degree UC patient (patient 3) carried a *CARD8* (rs2043211) mutation. While CD patient 2 was an exsmoker, the UC patient never smoked. In contrast to patient 1, patients 2 and 3 were both seronegative for the tested markers. The newly diagnosed fourth-degree relative was a female only 12 years old.

On average, the subjects at risk in the multicase families had 2.17 first-degree relatives with CD and 1.04 positive antibodies. Compared to the "average subject at risk," the odds to develop CD was thus 5.39 (95% CI: 2.99–9.73) for patient 1 but not elevated for patient 2 compared to the other subjects at risk. Since we did not investigate the odds

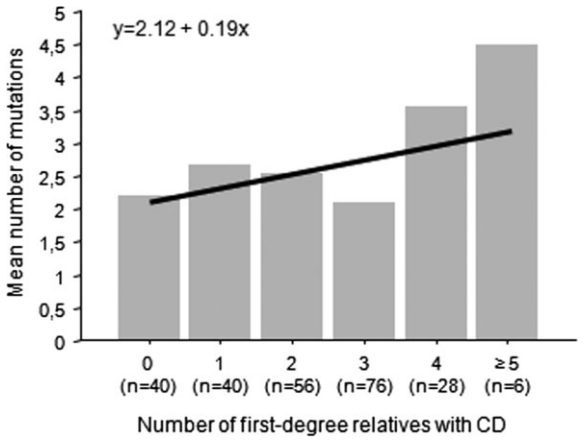


FIGURE 3. Mean number of mutations in unaffected family members from multicase families per number of first-degree relatives with CD.

TABLE 6. FBAT Allele Frequency Estimates and Informative Family Contribution

French Families		Original Frequencies	Original Number of Informative Families	Frequencies After Imputation	Number of Informative Families After Imputation
NOD2					
rs2066844	1	0.918	12	0.910	18
rs2066844	2	0.082	12	0.090	18
rs2066845	1	0.950	8	0.942	9
rs2066845	2	0.050	8	0.058	9
rs2066847	1	0.907	15	0.893	21
rs2066847	2	0.093	15	0.107	21
CARD8					
rs2043211	1	0.677	21	0.635	36
rs2043211	2	0.323	21	0.365	36
TLR4					
rs4986790	1	0.935	6	0.927	16
rs4986790	2	0.065	6	0.073	16
NOD1					
rs2907748	1	0.733	17	0.788	28
rs2907748	2	0.267	17	0.212	28
rs6958571	1	0.786	13	0.773	32
rs6958571	2	0.214	13	0.227	32
rs2075822	1	0.785	13	0.737	34
rs2075822	2	0.215	13	0.263	34
Belgian Families					
NOD2					
rs2066844	1	0.926	14	0.926	16
rs2066844	2	0.074	14	0.074	16
rs2066845	1	0.948	6	0.958	7
rs2066845	2	0.052	6	0.042	7
rs2066847	1	0.956	8	0.936	9
rs2066847	2	0.044	8	0.064	9
CARD8					
rs2043211	1	0.682	20	0.684	23
rs2043211	2	0.318	20	0.316	23
TLR4					
rs4986790	1	0.933	10	0.905	11
rs4986790	2	0.067	10	0.095	11
NOD1					
rs2907748	1	0.851	17	0.832	23
rs2907748	2	0.149	17	0.168	23
rs6958571	1	0.854	16	0.768	23
rs6958571	2	0.146	16	0.232	23
rs2075822	1	0.852	16	0.847	22
rs2075822	2	0.148	16	0.153	22

for developing UC in these families no comparison can be made for patient 3.

The defined interval (4.5 years) in a closed population (subjects at risk in the multicase families) allowed calculation of a precise incidence of IBD within the multiple-affected families, i.e., 0.008523. This incidence was 56.82-fold higher than the overall incidence of 10–15/100.000/year reported in our region.[36]

TABLE 7. Characteristics of the Newly Diagnosed First-degree Family Members

	Patient 1	Patient 2	Patient 3
Sex	Male	Male	Male
Disease	CD	CD	UC
Date of birth	11/04/1979	22/07/1962	25/02/1961
Date diagnosis	2002	2002	2005
Interval between sampling and diagnosis	24 months	13 months	54 months
Disease location at diagnosis	Ileocolitis	Ileitis	Left sided colitis
Disease behavior at diagnosis	Fistulizing	Inflammatory	Moderate
NOD2 mutations	rs2066844	Wildtype	Wildtype
CARD8 mutations	Wildtype	rs2043211	rs2043211
TLR4 mutations	Wildtype	Wildtype	Wildtype
NOD1 mutations	rs2075822,rs2907748, rs6958571	Wildtype	Wildtype
Smoking	Never	Exsmoker	Never
ASCA before diagnosis (>6)	9.60 (+)	1.90 (−)	4.30 (−)
ALCA before diagnosis (>90)	88.66 (−)	27.45 (−)	13.05 (−)
ACMA before diagnosis (>10)	0.29 (−)	2.21 (−)	6.87 (−)
AΣMA before diagnosis [ΣMan3 (>15) or ΣMan4 (>20)]	1.73 (−) 6.21 (−)	5.61 (−) 10.48 (−)	5.06 (−) 7.60 (−)
ACCA before diagnosis (>90)	51.38 (−)	44.11 (−)	15.75 (−)
I2 before diagnosis (>20)	10.05 (−)	13.66 (−)	7.66 (−)
OmpC before diagnosis (>23)	8.79 (−)	18.86 (−)	5.00 (−)
CBir1 Fla before diagnosis (>30)	60.05 (+)	13.11 (−)	11.08 (−)
Total positive antibodies before diagnosis	2	0	0
Total mutations	4	1	1
Number of first-degree relatives with CD	4	2	3

DISCUSSION

Over a period of 4½ years, we identified 4 new cases of IBD in 21 Belgian multiple-affected families as compared to none in matched simplex CD families or in healthy control families. Compared to the reported incidence of IBD of 10–15/100.000/year in our region, these 4 new cases revealed a 57-fold increase in incidence. Unaffected relatives in multicase families are therefore at a much higher risk to develop IBD. In this study we investigated the usefulness of potential predictive factors but the unexpected high incidence of IBD within these multiple-affected families could not be explained based on the studied markers. None of the families where new diagnoses occurred in first-degree relatives expressed an excess of genetic or serologic markers. A very high genetic load (mean number of mutations 5.14) was present only in the family where a diagnosis of CD was made in a fourth-degree relative. The first-degree relative who was diagnosed with CD at age 23 (i.e., patient 1), carried 4 genetic risk factors (1 mutation in *NOD2*, 3 mutations in *NOD1*). The 2 remaining first-degree patients who were diagnosed with IBD at age 40 and 44 only carried 1 of the studied genetic risk factors (a mutation in *CARD8*). Although genetic and serologic risk factors were present overall, there were no specific disease-causing combinations observed. Also, when comparing the multicase families with the single-case families, no clear discriminating factors were found, suggesting that the gene-environment interactions might not occur simultaneously between families and/or might be specific to each family.

When calculating relative risk for CD in the multi-affected families, the effect of affected sibships was confirmed and a cumulative effect of the number of positive antibodies was detected as well. Since multicase families were identified through patients with known affected relatives, these probands were excluded from the analyses to make sure the measured effect was not due to the study design but to a true finding. The calculated odds with regard to the number of first-degree relatives and number of positive antibodies are thus probably even an underestimation for subjects from multicase families. An increased risk for CD was found per additional first-degree relative with CD and cumulative per additional positive antibody (Table 4), which might subsequently enhance the risk stratification.

Overall, patients on average expressed a higher load of antibodies as compared to unaffected subjects. Although the unaffected relatives also expressed antibodies, one

could hypothesize that they probably do not reach the threshold to present clinically with disease. This raises the question whether antibodies could help to differentiate between subjects at risk and subjects who will never be affected with CD.

The novel patients are all from multicase families, confirming the risk effect of number of first-degree relatives with CD. Since we found that the expression of antibodies was an important risk factor for developing CD in these families, it is possible that the blood samples were taken before increased immunologic response to microbial antigens reached the arbitrary threshold for antibody positivity. In patient 1, for example, the measured antibody titer for ALCA was just below this threshold (88.66 with threshold 90). However, in order to prove this reassessment of the antibodies status after diagnosis should have been done. It has been shown that patients with CD express ASCA the before time of diagnosis.[23] In that study by Israeli et al, the mean interval between measurement of antibodies and the diagnosis was 38 months. Only 1 of the newly diagnosed CD patients expressed ASCA positivity, 24 months before diagnosis. For 2 of the other new first-degree patients, the interval between sampling and diagnosis was 13 and 54 months, respectively. Given that the individual sensitivity of serologic markers is low and, furthermore, not knowing the timepoints for assessment of seroreactivity, the utility of these markers in risk assessment is doubtful.

Although we expected risk factors for CD to cluster within multiple-affected families, we showed a significant correlation between the number of genetic mutations and an increasing number of first-degree relatives with CD ($P = 0.0195$). No such positive correlation between the number of antibodies and the number of first-degree relatives could be found, however. Since the unaffected subjects in our cohort have up to 7 first-degree relatives with CD, the association between the number of first-degree relatives with CD and genetic determinants of the disease demonstrates once more that CD is a true multifactorial disease, in which other factors besides genetic predisposition may play a mandatory pathophysiological role.

Overall, we demonstrated that the genetic load is heavier in subjects in the presence of a larger number of first-degree relatives affected with CD. For serological markers we found that the average intensities of the antibodies in the relatives of sporadic patients was slightly but significantly higher than those of relatives of familial patients (averaged mean sum of quartiles scores was 2.43 versus 2.21, respectively). However, none of the studied genetic markers nor the serologic markers could discriminate between single-case families or multicase families.

The novel finding that an increment of risk for CD per additional first-degree relative with CD and per additional positive antibody is present in multicase families further supports the hypothesis that antimicrobial antibody formation in CD might play a key role in the prediction of subjects at risk in those families, however. Larger longitudinal studies are now eagerly awaited to confirm our findings.

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