IBD1 and IBD3 Determine Location of Crohn's Disease in the Spanish Population

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Background: Crohn's disease is a heterogeneous disease from both genetic and clinical points of view.

Aims: To look for associations between distinct genetic polymorphisms and clinical subgroups of the disease.

Subjects: A total of 210 patients and 343 healthy control subjects, all adult, unrelated, white, Spanish individuals.

Methods: DNA was purified from peripheral blood samples and was typed by sequence-specific oligonucleotide polymerase chain reaction (PCR) method for human leukocyte antigen (HLA)-DRB1 alleles (IBD3) and by allele-specific PCR for *NOD2/CARD15* (IBD1) polymorphisms.

Results: *NOD2/CARD15* mutations and HLA-DRB1*07 confer susceptibility only to the ileal location of the disease, whereas HLA-DRB1*0103 is associated only with the colonic location of the disease. The IBD3 effect was overshadowed by IBD1 mutations when present.

Conclusion: The studied genetic polymorphisms of Crohn's disease basically determine the location of the disease and, only secondarily, the clinical form of the disease. This appears to be true for both inflammatory bowel diseases as HLA-DRB1*0103 is associated both with colonic Crohn's disease and ulcerative colitis.

Key Words: Crohn's disease, human leukocyte antigen-DRB1, *NOD2/CARD15*, ulcerative colitis

(*Inflamm Bowel Dis* 2004;10:715–722)

Received for publication March 28, 2003; accepted April 28, 2004.

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This work was supported by Grant SAF 2003-08522 and from the Alfonso Martin Escudero Foundation.

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Crohn's disease is a chronic inflammatory disorder of unknown origin involving various sites in the gastrointestinal tract. Several environmental, microbial, immunologic, genetic, and life-style factors have been suggested to play a role in the initiation of the disease.[1,2] Heterogeneity is observed in terms of disease location, behavior, age of onset, and surgical history. There is some support for the concept that this heterogeneity may be in large measure genetically determined.[3–6]

Genome-wide scans performed in patients with inflammatory bowel disease (IBD) have failed to find a major unique susceptibility locus and have prompted the general agreement that Crohn's disease is a polygenic entity in which several genes may contribute to susceptibility. The major IBD susceptibility loci identified, demonstrating significant or suggestive evidence for linkage, have been on chromosomes 16 (IBD1), 12 (IBD2), 6 (IBD3, the human leukocyte antigen [HLA] region), 14 (IBD4), 5 (IBD5), 19 (IBD6), 1, 7, and 3.[7–16]

Several groups have recently reported associations between the *NOD2/CARD15* gene, which is located in human chromosome 16q (IBD1), and susceptibility to Crohn's disease.[17–19] A frameshift mutation (Leu1007fsinsC) and two nonsynonymous single-nucleotide polymorphisms (SNPs Arg702Trp and Gly908Arg), which were found in 30% of the patients, were significantly associated with the disease overall. Several studies also have reported an association between *NOD2/CARD15* and ileal/fibrostenosing disease.[20–24]

The HLA region (IBD3) on chromosome 6 contains more than 200 genes, many of which participate in the regulation of immune and inflammatory responses, therefore providing ideal candidates for etiologic investigations. IBD3 on chromosome 6 has been involved mainly in susceptibility to ulcerative colitis,[25–27] whereas the results in Crohn's disease in white patients have been contradictory, and some important studies have not found any overall association.[25] In other instances, positive associations with HLA-DRB1*01,[28,29] which were later attributed to the DRB1*0103 subtype,[20,30] DRB1*1302[31–33], and DRB1*07,[20,29,32,34] and negative associations with HLA-DR2,[20,32,35] which were attributed to the DRB1*1501 subtype,[20,35] and with HLA-DRB1*03[29,32,34]

have been reported. The results of previous studies of the HLA class II genes produced conflicting data, probably due to methodological and ethnic differences, as well as disease heterogeneity. Besides, the linkage disequilibrium seen across the HLA region results in highly conserved haplotypes, making the interpretation of results difficult. Other genes or markers (ie, tumor necrosis factor, major histocompatibility complex class I chain-like gene A, and HSP-70) in addition to HLA-DR have also been reported to be associated with Crohn's disease.[36–38]

As HLA genes appear to confer only a modest risk of Crohn's disease overall, we speculated that associations might be stronger or present only in specific patient subgroups. To examine this hypothesis, the patient population was stratified by phenotype, as defined by the Vienna classification,[39] and on the basis of the presence or absence of the *NOD2/CARD15* mutations to look for susceptibility genes being present in only one or some of these subpopulations. The study was performed in two steps. First, we performed a study of HLA class II DRB1 genes in Crohn's disease patients overall. Second, we stratified the patients into those patients who were positive or negative for any of the three *NOD2/CARD15* gene polymorphisms.

MATERIALS AND METHODS

Patients and Control Subjects

The study group consisted of 210 adult, unrelated, white Spanish patients (53% women; median follow-up 9.9 years; follow-up range 1–44 years) who had been recruited from a single center. The diagnosis of Crohn's disease was based on standard clinical, radiologic, endoscopic, and histologic data following the criteria of Lennard-Jones.[40] Patients and data are regularly followed up in the Inflammatory Bowel Disease Unit at San Carlos University Hospital, Madrid. Phenotypic details were obtained from clinical histories and personal interviews of patients. Disease phenotype was determined following the Vienna classification.[39] Locations were designated as follows: L1 (terminal ileum); L2 (colonic); L3 (ileocolonic); and L4 (upper gastrointestinal). Behavior was designated as follows: B1 (inflammatory, nonstricturing, nonpenetrating); B2 (stricturing); and B3 (penetrating). Perianal disease was defined by the presence of perianal abscesses, fistulas, and/or ulcers. A group of 343 healthy, white, unrelated subjects (61% women) from the Madrid region (mainly hospital employees and blood donors) were selected as control subjects.

DNA Isolation and HLA Class II Genotyping

DNA was extracted from fresh peripheral blood leukocytes by a "salting out" procedure with 6 mol/L NaCl after overnight incubation with proteinase K.[41] The hypervariable second exon of the HLA-DRB1 locus was amplified by a polymerase chain reaction (PCR) procedure using the appropriate primers from the 11th International Histocompatibility Workshop.[42] The quality of PCR product was assessed by agarose gel electrophoresis. From the PCR products, 5 μL (about 20 ng) was slot-blotted onto nylon membranes and cross-linked by exposure to ultraviolet light. Hybridization was performed with digoxygenin-labeled, allele-specific oligonucleotide probes from the 11th Histocompatibility Workshop to characterize the DRB1 allele groups. The results of this sequence-specific oligonucleotide PCR technique were visualized by exposing the membranes to radiographic films for 5 to 15 minutes.

HLA-DR1, HLA-DR2-, HLA-DR4-, HLA-DR11-, and HLA-DR6-positive DNAs, identified in the HLA-DRB1 generic PCR and hybridization, were then specifically typed for the individual alleles within the HLA-DR1, HLA-DR2, HLA-DR4, HLA-DR11, or HLA-DR6 groups by amplification in the HLA-DRB1 group-specific PCR and by hybridization with a panel of sequence-specific oligonucleotide probes from the 11th International Histocompatibility Workshop.[42]

NOD2/CARD15 Polymorphisms

SNP13 (Leu1007fsinsC) was genotyped using a TaqMan assay (Applied Biosystems, Foster City, CA). The primers and probes used have been described previously,[19] and the PCR products were analyzed in an ABI 7700 Sequence Detector (Applied Biosystems). SNP8 (Arg702Trp) (sense, 5′-CAT CTG AGA AGG CCC TGC TC(C/T)-3′; antisense, 5′-CAG ACA CCA GCG GGC ACA-3′) and SNP12 (Gly908Arg) (sense, 5′-TTG GCC TTT TCA GAT TCT GG (G/C)-3′; antisense, 5′-CCC CTC GTC ACC CAC TCT G-3′) were typed by allele-specific PCR. The detection of a wild-type/mutant was assessed in an ABI 7700 Sequence Detector by a SyberGreen assay. Previously sequenced samples were used as controls. In cases of doubt, samples were sequenced to confirm the result.

Statistical Analysis

The allele carriage rates in patients and control subjects were compared by the χ^2 test or Fisher exact test when an expected value was <5, and *P* values were considered to be significant at a level of <0.05. For HLA-DRB1 genotyping, the Bonferroni correction was applied. For those DRB1 alleles the results of which have already been reported, no correction has been applied. Odds ratios (ORs), 95% confidence intervals (CIs), and *P* values were calculated using a standard informatic package (Epi Info, version 6.02; CDC, Atlanta, GA).

RESULTS

General Analysis

Disease Susceptibility

We examined HLA-DR class II alleles. A negative association of alleles HLA-DR2 (15.7%; healthy control subjects 26.0%; $P = 0.004$; OR 0.53) and the DR2 subtype DRB1*1501 (11.4%; healthy control subjects 18.6%; $P = 0.02$; OR 0.56;

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TABLE 1. Distribution of DRB1 Alleles† Among the Different Clinical Subgroups of Crohn's Disease

	n	DRB1*1501 Allele	DRB1*07 Allele	DRB1*0103 Allele	DRB1*1302 Allele	DRB1*03 Allele
Disease susceptibility						
Crohn's disease	210	24[a]	74[b]	14[d]	25	48
Control subjects	343	64	92	7	27	82
Disease location						
Ileal (L1)	97	10	37[c]	1	13	23
Colonic (L2)	36	3	7	10[e]	5	7
Ileocolonic (L3)	69	11	27	3	6	16
Upper GI tract (L4)	8	0	3	0	1	2
Disease behavior						
Inflammatory (B1)	89	7	35	5	9	19
Stricturing (B2)	35	2	14	1	6	11
Perforating (B3)	86	15	25	8	10	18
Perianal	45	7	8	7[f]	3	7
Not Perianal	165	17	66	7	22	41

Only the most relevant DRB1 alleles for Crohn's disease are shown. Values are given as the number of patients. GI, gastrointestinal.

[a]Allele DRB1*: Crohn's disease versus control subjects, $P = 0.02$, OR 0.56.

[b]Allele DRB1*07: Crohn's disease versus control subjects, $P = 0.03$, OR 1.48.

[c]Allele DRB1*07: ileal disease (L3) versus colonic disease (L2); $P = 0.03$, OR 2.6.

[d]Allele DRB1*0103: Crohn's disease versus control subjects, $P = 0.005$, OR 3.43.

[e]Allele DRB1*0103: colonic disease (L2) versus ileal disease, $P < 10^{-6}$, OR 40.

[f]Allele DRB1*0103: perianal disease versus no perianal disease, $P = 0.007$, OR 4.16.

95% CI 0.33–0.96) was observed in Crohn's disease patients. Allele DRB1*07 showed a positive association with Crohn's disease (35.2%; healthy control subjects 26.8%; $P = 0.03$; OR 1.48; 95% CI 1.01–2.19), as did allele DRB1*0103 (6.6%; healthy control subjects 2.0%; $P = 0.005$; OR 3.43; 95% CI 1.27–9.55) (Table 1).

Disease Location

Location is a stable determinant of phenotype in Crohn's disease. Patients were classified according to the location of the lesions in four groups: terminal ileum (L1), 46.2% of patients; colonic (L2), 17.1% of patients; ileocolonic (L3), 32.8% of patients; and upper GI tract (L4), 3.8% of patients (Table 2). As all L4 patients also had lesions in the terminal ileum, in the statistical analysis they were considered to be part of the same group (designated Li from this point; Li = L1 + L4).

HLA DRB1*1501 showed no significant differences between groups. HLA DRB1*0701 was positively associated with the presence of ileal disease, irrespective of the presence of colonic disease, when compared with healthy control subjects (Li 38.1% [$P = 0.03$; OR 1.68]; L3 39.1% [$P = 0.04$; OR 1.75]; versus control subjects 26.8%; Li + L3 38.5%; control subjects 26.8%; $P = 0.007$; OR 1.71; 95% CI 1.14–2.56). The difference was even greater between ileal and colonic patients (Li + L3 38.5%; L2 19.4%; $P = 0.03$; OR 2.6; 95% CI 1.01–6.92) (Table 1).

HLA-DRB1*0103 was significantly associated with the presence of colonic disease (L2) when compared with control subjects (27.7% versus 2.0%, respectively; $P < 10^{-6}$; OR 18.46; 95% CI 5.86–59.52). HLA-DRB1*0103 was also more frequent in patients with ileocolonic disease (L3) than in healthy control subjects or in those with ileal (Li) disease (L3 4.3%; control subjects 2.0%; Li 0.95%), but the differences were not statistically significant. The biggest difference in DRB1*0103 frequency was found between patients with colonic (L2) and ileal (Li) disease (27.7% versus 0.95%, respectively; $P < 10^{-6}$; OR 40; 95% CI 4.87–873.04) (Table 1).

DRB1*0103 was also found to be associated with perianal disease when compared with healthy control subjects (15.5% versus 2.0%, respectively; $P = 0.000005$; OR 8.84; 95% CI 2.61–30.00) and compared with no perianal disease (15.5% versus 4.2%, respectively; $P = 0.007$; OR 4.16; 95% CI 1.22–14.22) (Table 1).

As perianal lesions were frequently present in patients with colonic disease (30.6%), we performed a stratified analysis to determine whether allele DRB1*0103 was primarily associated with colonic and/or perianal disease. Allele DRB1*0103 was significantly increased in colonic patients (both L2 and L2

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TABLE 2. Clinical Features and Carriage of *NOD2/CARD15* Mutations in All 210 Crohn's Disease Patients

Crohn's Disease (n = 210)	L1 (n = 97)	L2 (n = 36)	L3 (n = 69)	L4 (n = 8)	Perianal (n = 45)
B1 (n = 89)	43	21	25	0	0
Nod2+ (n = 22)	15	2	5		0
Nod2− (n = 67)	28	19	20		0
B2 (n = 35)	27	1	4	3	0
Nod2+ (n = 16)	14	0	1	1	
Nod2− (n = 19)	13	1	3	2	
B3 (n = 86)	27	14	40	5	45
Nod2+ (n = 28)	12	2	12	2	12
Nod2− (n = 58)	15	12	28	3	33

Nod2+, positive for any of the three *NOD/CARD15* variants (SNP8, SNP12, SNP13); Nod2−, negative for all three *NOD2/CARD15* variants (SNP8, SNP12, SNP13).

+ L3 patients) with no perianal lesions (L2 20%; control subjects 2.0%; $P = 0.0005$; OR 12; 95% CI 2.98–47.51; L2 + L3 9.1%; control subjects 2.0%; $P = 0.01$; OR 4.8 95% CI 1.37–16.60), but not in Li patients with perianal lesions (Li 0%; healthy control subjects 2.0%; difference was not significant) (Table 3).

Clinical Behavior

Each patient was also defined by clinical behavior: B1 was present in 42.4% of patients; B2 was present in 16.6% or patients; and B3 was present in 40.9% of patients (Table 2).

The frequency of allele DRB1*1501 was decreased in patients with inflammatory lesions when compared with control subjects (7.9% versus 18.6%, respectively; $P = 0.01$; OR 0.37), but no statistically significant differences were found between the different clinical behaviors.

The frequency of allele DRB1*0701 was increased in the three groups but only reached statistical significance in patients with inflammatory lesions when compared with control subjects (39.3% versus 26.8%, respectively; $P = 0.02$; OR 1.77).

Penetrating disease was associated with allele DRB1*0103 when compared with control subjects (9.3% versus 2.0%, respectively; $P = 0.001$; OR 4.92; 95% CI 1.57–15.63), although the difference was not statistically significant when compared with the other groups (Table 1).

As penetrating disease was common among colonic patients, we performed a stratified analysis to determine whether DRB1*0103 was primarily associated with penetrating disease or was secondary to the presence of colonic disease. The frequency of allele DRB1*0103 was significantly increased in colonic patients (both L2 and L2 + L3 patients) with no penetrating lesions (L2 patients 18.2%; control subjects 2.0% [$P = 0.00001$; OR 10.67; 95% CI 2.36–46.17]; L2 + L3 patients 9.8%; healthy control subjects 2.0% [$P = 0.002$; OR 5.22; 95% CI 1.37–19.33]) but not in ileal patients with penetrating lesions (L1 0%; healthy control subjects 2.0%; difference not significant) (Table 3).

Alleles DRB1*1302 and DRB1*03 were not associated with Crohn's disease overall or with any specific location or behavior.

Stratification by *NOD2/CARD15* Gene Mutations

Of the 210 patients with Crohn's disease who were studied, 66 patients (31.4%) carried at least one of the three *NOD2/CARD15* mutations (SNP8 14.7%; SNP12 7.6%; SNP13 12.8%), and 144 patients possessed no variant (Table 2). A total of 9.3% of the healthy control subjects carried one of the three *NOD2/CARD15* mutations (SNP8 5.4%; SNP12 2.3%; SNP13 1.6%). Ileal disease (Li) was present more frequently in *NOD2/CARD15*-positive patients than in *NOD2/CARD15*-negative patients (66.6% versus 42.3%, respectively; $P = 0.001$; OR 2.72; 95% CI 1.42–5.25), whereas colonic disease (L2) was present less frequently (6% versus

TABLE 3. DRB1*0103 Allele Distribution Among Colonic, Penetrating, and/or Perianal Patients

	Ileal (Li)	Colonic (L2)	Ileocolonic (L3)	L2 + L3
Perianal	0/6 (0%)	5/11 (45.4%)	2/28 (7.1%)	7/39 (17.9%)
Not perianal	1/99 (1%)	5/25 (20%)[a]	1/41 (2.4%)	6/66 (9.1%)[b]
Penetrating	0/32 (0%)	6/14 (42.9%)	2/40 (5%)	8/54 (14.8%)
Not penetrating	1/73 (1.3%)	4/22 (18.2%)[c]	1/29 (3.4%)	5/51 (9.8%)[d]

[a]L2 colonic not perianal vs control subjects: $P = 0.0005$; OR 12; 95% CI 2.98–47.51.
[b]L2L3 colonic not perianal vs control subjects: $P = 0.01$; OR 4.8; 95% CI 1.37–16.60.
[c]L2 colonic not penetrating vs control subjects: $P = 0.00001$; OR 10.67; 95% CI 2.36–46.17.
[d]L2L3 colonic not penetrating vs control subjects: $P = 0.002$; OR 5.22; 95% CI 1.37–19.33.

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22%, respectively; $P = 0.004$; OR 0.23; 95% CI 0.06–0.71). Stricturing disease (B2) was also more frequent in *NOD2/CARD15*-positive patients than in *NOD2/CARD15*-negative patients (24.2% versus 13.2%, respectively; $P = 0.04$; OR 0.04; 95% CI 0.94–4.70) (Table 4).

Disease Susceptibility

When we examined the association of DRB1 HLA class II alleles with *NOD2/CARD15* mutation-positive and mutation-negative patients separately, no new positive or negative association was found. The negative association of DRB1*1501 was observed in *NOD2/CARD15*-negative patients when compared with healthy control subjects (9.7% versus 18.6%, respectively; $P = 0.01$; OR 0.47), but not in *NOD2/CARD15*-positive patients (15.1% versus 18.6%, respectively). No statistically significant difference in DRB1*1501 frequency was found between *NOD2/CARD15*-positive and *NOD2/CARD15*-negative patients.

Allele DRB1*0701 presented a higher frequency both in *NOD2/CARD15* mutation-negative (34%) and *NOD2/CARD15* mutation-positive patients (37.8%) when compared with control subjects (26.8%), although the differences did not reach

TABLE 4. Distribution of DRB1 Alleles Among the Different Clinical Subgroups of Crohn's Disease Stratified by *NOD2/CARD15* Status

	n	DRB1*1501 Allele	DRB1*07 Allele	DRB1*0103 Allele
Crohn's disease *NOD2/CARD15*-negative	144	14[a]	49	13[d,e]
Disease location				
Ileal (L1)	56	5	21[b]	0
Colonic (L2)	32	3	7	10[f,g]
Ileocolonic (L3)	51	6	19	3
Upper GI tract (L4)	5	0	2	0
Disease behavior				
Inflammatory (B1)	67	5	27	5
Stricturing (B2)	19	2	8	0
Perforating (B3)	58	7	14	8
Perianal	33	4	5	7[h]
Not perianal	111	10	44	6
Crohn's disease *NOD2/CARD15*-positive	66	10	25	1
Disease location				
Ileal (L1)	41	5	16[c]	1
Colonic (L2)	4	0	0	0
Ileocolonic (L3)	18	5	8	0
Upper GI tract (L4)	3	0	1	0
Disease behavior				
Inflammatory (B1)	22	2	8	0
Stricturing (B2)	16	0	6	1
Perforating (B3)	28	8	11	0
Perianal	12	3	3	0
Not perianal	54	7	22	1
Control subjects	343	64	92	7

Only the most relevant DRB1 alleles for Crohn's disease are shown. Values are given as the number of patients. See Table 2 for abbreviations not used in the text.

[a]Allele DRB1*1501: Crohn's disease versus control subjects, $P = 0.01$, OR 0.47.

[b]Allele DRB1*07: ileal disease (L1 + L3) versus control subjects, $P = 0.03$, OR 1.63.

[c]Allele DRB1*07: ileal disease (L1 + L3) versus control subjects, $P = 0.03$, OR 1.87.

[d]Allele DRB1*0103: Crohn's disease versus control subjects, $P = 0.0004$, OR 4.76.

[e]Allele DRB1*0103: Crohn's disease Nod2-negative versus Crohn's disease Nod2-positive, $P = 0.04$, OR 6.45.

[f]Allele DRB1*0103: colonic disease (L2) Nod2-negative versus control subjects, $P < 10^{-6}$, OR 21.82.

[g]Allele DRB1*0103: colonic disease (L2) versus ileal (L1 + L3) disease, $P < 10^{-6}$, OR 16.52.

[h]Allele DRB1*0103: perianal versus not perianal disease, $P = 0.005$, OR 4.71.

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statistical significance. The presence of both *NOD2/CARD15* mutation and DRB1*07 in one individual did not increase the risk for disease compared with the presence of the *NOD2/CARD15* mutation alone (*NOD2/CARD15*-positive-DRB1*07-positive: OR 4.32; 95% CI 1.48–13.51; *NOD2/CARD15*-positive-DRB1*07-negative: OR 8.85; 95% CI 2.87–30.44).

Allele DRB1*0103 was associated with the disease in *NOD2/CARD15* mutation-negative patients compared to control subjects (9% versus 2%, respectively; $P = 0.0004$; OR 4.76; 95% CI 1.73–13.52), but not in *NOD2/CARD15* mutation-positive patients (1.5%). There was also a significant difference between *NOD2/CARD15* mutation-negative and *NOD2/CARD15* mutation-positive patients (9% versus 1.5%, respectively; $P = 0.04$; OR 6.45) (Table 4).

Disease Location

The negative association with DRB1*1501 showed no differences between groups. HLA DRB1*0701 was positively associated with the presence of ileal disease, with or without colonic disease, in both *NOD2/CARD15*-negative and *NOD2/CARD15*-positive patients (37.7% and 38.5%, respectively; control subjects 26.8%). No additive effect was found between these two genomic regions for ileal lesions, as these were found with the same increased frequency in *NOD2/CARD15*-positive-DRB1*07-positive patients (68%) and *NOD2/CARD15*-positive-DRB1*07-negative patients (66%). The risk for ileal lesions was not so marked in *NOD2/CARD15*-negative-DRB1*07-positive patients compared to all *NOD2/CARD15*-positive patients (47% versus 67%, respectively; $P = 0.03$; OR 0.44).

The presence of HLA DRB1*0103 was increased in *NOD2/CARD15*-negative patients with colonic disease compared with control subjects (31.3% versus 2%, respectively; $P < 10^{-6}$; OR 21.82; 95% CI 6.81–71.60) and when compared with those patients with ileal disease (L2 31.3%; L1 + L3 2.7%; $P = 10^{-6}$; OR 16.52; 95% CI 3.74–83.30) (Table 4). Associations between DRB1*0103 and colonic disease in the *NOD2/CARD15*-positive group were difficult to ascertain, due to the very small number of patients with this location of the lesions. However, the only DRB1*0103-positive patient in the *NOD2/CARD15*-positive group had an ileal stricturing disease and no colonic lesions.

The frequency of DRB1*0103 was also significantly higher in *NOD2/CARD15*-negative patients with perianal disease when compared with healthy control subjects or with patients without perianal disease (21.2% versus 5.4%, respectively; $P = 0.005$; OR 4.71; 95% CI 1.28–17.61). As previously shown for the totality of patients, the association of DRB1*0103 with perianal disease was secondary to colonic lesions also in the *NOD2/CARD15*-negative group (data not shown).

Clinical Behavior

Distribution of HLA DRB1*1501 or DRB1*0701 showed no significant differences in any of the three clinical behaviors between *NOD2/CARD15*-positive and *NOD2/CARD15*-negative patients.

DRB1*0103 was associated with penetrating disease in *NOD2/CARD15*-negative patients when compared with healthy control subjects (13.8% versus 2%, respectively; $P = 10^{-6}$; OR 7.68), but differences were not significant when compared with the other subgroups. Again, the association of penetrating disease with DRB1*0103 was secondary to that with colonic lesions in the *NOD2/CARD15*-negative group (data not shown).

DISCUSSION

Our results have not detected any new association between HLA genes and Crohn's disease, but they showed that a strong relation exists between disease location and genetic heterogeneity.

They confirmed the association between *NOD2/CARD15* mutations and ileal disease,[20–24] and the strong association between DRB1*0103 allele and colonic disease.

Of the associations highlighted on a recent meta-analysis,[32] our results were consistent with the positive association with HLA-DRB1*07 and the negative association with DR2 (DRB1*1501), but they did not show a decreased frequency of the allele DRB1*03 or an increased frequency of DRB1*1302.

When stratified analyses were performed, the only significant difference with the DRB1*1501 allele was that its protective role was only observed in the *NOD2/CARD15*-negative group.

Similar to the results of Ahmad et al,[20] the most significant differences for DRB1*07 and DRB1*0103 were seen when patients were stratified on the basis of disease location. HLA-DRB1*07 was associated with patients with ileal (L1) and ileocolonic (L3) diseases, whereas DRB1*0103 was much more frequent in patients with colonic disease (L2). When stratified by clinical behavior, the former allele was not associated with any subgroup, and the latter was associated with penetrating lesions, but this was seen to be secondary to the association between this behavior and the disease location (colonic).

Silverberg et al[43] have recently described an association between DRB1*0103 and colonic location, and our data show that this occurs in *NOD2/CARD15*-negative individuals, although this should be confirmed in a larger group of patients.

We studied the possible interactions between susceptibility loci located within chromosome 16 (IBD1) and within chromosome 6 (IBD3). No additive effect was found between these two regions for susceptibility to Crohn's disease nor for the presence of ileal lesions, as these were found with the same increased frequency in *NOD2/CARD15*-positive-DRB1*07-positive patients and in *NOD2/CARD15*-positive-DRB1*07-

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negative patients. Although both *NOD2/CARD15* mutations and DRB1*0103 were associated with Crohn's disease, no additive effect was observed. This stressed the genetic heterogeneity of the disease as the former polymorphism conferred susceptibility to ileal lesions and the latter to colonic lesions. Moreover, even if the frequency of each of those two markers (*NOD2/CARD15* and DRB1*0103) was increased in patients, the presence of *NOD2/CARD15*-positive-DRB1*0103-positive individuals was not (1/210 patients or 0.48%). Furthermore, the only *NOD2/CARD15*-positive-DRB1*0103-positive patient had an ileal stricturing disease without colonic lesions.

Therefore, we can conclude that there is an association between several HLA-DRB1 alleles in the IBD3 region and susceptibility to Crohn's disease:

- HLA-DRB1*1501 is a protective allele for the disease overall.
- HLA-DRB1*07 confers susceptibility to the ileal form of the disease, with (L3) or without colonic lesions (Li).
- HLA-DRB1*0103 confers susceptibility to the colonic form of the disease (L2).

NOD2/CARD15 mutations confer susceptibility to the ileal form of the disease, and in their presence HLA-DRB1*1501 was not seen to be protective, HLA-DRB1*07 was not seen to confer an additive effect, and HLA-DRB1*0103 was not seen to confer additive susceptibility to the colonic form of the disease.

Clinical behavior in patients with ileal disease is partly dependent on the genetics: *NOD2/CARD15*-positive ileal patients have stricturing lesions more frequently, whereas *NOD2/CARD15*-negative-DRB1*07-positive patients with ileal disease have inflammatory (ie, nonstricturing, nonpenetrating) lesions more frequently. Also, the association between DRB1*0103 and the penetrating behavior appears to be secondary to the presence of colonic and perianal lesions found in patients carrying this allele. It is interesting to note that DRB1*0103 is strongly associated both with ulcerative colitis[25–27] and Crohn's disease located exclusively in the colon, and that these two inflammatory colonic diseases therefore appear to be genetically related. This location of Crohn's disease (L2) was seen to have a quite different genetic basis than those of the other locations (ie, *NOD2/CARD15*-negative, DRB1*0103-positive, and DRB1*07-negative patients) and usually has a distinct clinical behavior.

Having been established the genetic heterogeneity of IBD and its importance in the location of the disease, and secondarily its clinical form, it would be interesting to study whether other phenotypic manifestations (eg, treatment response) also show such complex genetic profiles.

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