ORIGINAL ARTICLE

Taylor & Francis
healthsciences

Prevalence of Mutations of the *NOD2/CARD15* Gene and Relation to Phenotype in Spanish Patients with Crohn Disease

J. L. Mendoza, L. S. Murillo, L. Fernández, A. S. Peña, R. Lana, E. Urcelay, D. M. Cruz-Santamaría, E. G. de la Concha, M. Díaz-Rubio & J. García-Paredes
Depts. of Gastroenterology and Immunology, Hospital Clínico San Carlos, Universidad Complutense, Madrid, Spain; Dept. of Immunology, Gastroenterology and Laboratory of Immunogenetics, Vrije Universiteit Medical Centre, Amsterdam, The Netherlands

Mendoza JL, Murillo LS, Fernández L, Peña AS, Lana R, Urcelay E, Cruz-Santamaría DM, de la Concha EG, Díaz-Rubio M, García-Paredes J. Prevalence of mutations of the *NOD2/CARD15* gene and relation to phenotype in Spanish patients with Crohn disease. Scand J Gastroenterol 2003;38:1235–1240.

Background: We assessed the prevalence of R702W, G908R, and L1007fs coding mutations in the *NOD2/CARD15* gene and the genotype–phenotype relation in Spanish patients with Crohn disease. **Methods:** A cohort of 204 unrelated patients with Crohn disease and 140 healthy controls were studied. The phenotype was established before commencement of genotyping. Genotyping of the R702W, G908R, and L1007fs gene polymorphisms of *NOD2/CARD15* was performed by two independent laboratories using different techniques. In the case of discordant results, specific sequencing of DNA strands was performed. **Results:** At least one mutation was present in 32.8% of patients compared to 10.7% in controls (OR = 4.08, 95% CI 2.21 to 7.50). In patients with Crohn disease, the frequency of R702W, G908R, and L1007fs carriers was 13.7%, 8.3%, and 14.2%, respectively. Compound heterozygotes and homozygotes occurred in 3.4% and 2.9% of patients and in none of the controls. The correlation of genotype–Vienna classification showed a significant association with ileal disease (RR = 1.61, 95% CI 1.21–2.15, $P = 0.001$) and an inverse association with colonic localization (RR = 0.29, 95% CI 0.11–0.80, $P = 0.007$). There was a significant association between G908R carriership and previous appendectomy, surgical interventions, and stricturing behavior. A gene-dosage effect on phenotypic characteristics was not observed. **Conclusions:** In a Spanish population from Madrid, mutations of the *NOD2/CARD15* gene were a marker of susceptibility to Crohn disease and were associated with ileal disease. Carriers of the G908R mutation showed a stricturing disease behavior, history of appendectomy, and surgical interventions over the course of the disease.

Key words: CARD15/NOD2 gene; Crohn disease; Vienna classification

Juan Luis Mendoza, Servicio de Aparato Digestivo, Unidad de Enfermedad Inflamatoria Intestinal, Hospital Clínico San Carlos de Madrid, Martín Lagos s/n, ES-28040 Madrid, Spain (fax. +34 91 330 3785, e-mail. jmendozah@meditex.es)

Crohn disease is a chronic inflammatory disorder of the gastrointestinal tract. The inflammation may involve any segment of the digestive tract, from the mouth to the anus, and may affect the mucosa and the deeper layers of the digestive wall, with or without granulomas. The etiopathogenesis of the disease remains poorly understood. Experimental and observational data suggest that intestinal inflammation arises from abnormal immune reactivity to bacterial flora in the intestine of individuals who are genetically susceptible (1).

The combined strategies of positional cloning and candidate gen analysis of chromosome 16 have recently led three independent groups to identify *NOD2*—the nomenclature of *NOD2* has been changed to *CARD15*—as a gene linked to Crohn disease (2–4). The *NOD2/CARD15* gene is located at the Crohn disease susceptibility locus (*IBD1*) on chromosome 16q12. The respective gene product is expressed in monocytes and intestinal epithelial cells (5, 6), functions as an intracellular receptor for bacterial components, and is involved in apoptosis and nuclear factor κB (NFκB) activation, which is a key transcriptional factor involved in initiation of immunoinflammatory responses (7).

It has been demonstrated that three single-nucleotide polymorphisms (SNPs) R702W, G908R, L1007fs (also called SNP8, SNP12, and SNP13, respectively) within the *NOD2/CARD15* gene are associated with Crohn disease. On the other hand, it has been suggested that various disease phenotypes including age at diagnosis, sex, family history, location of disease, response to treatment, and behavior of disease may be gentically determined (8). In fact, recent studies have provided a link between *NOD2/CARD15* mutations and clinical characterization of Crohn disease (9–15). Marked racial differences are observed for common Crohn disease-associated variants. The frequency of mutant *NOD2/CARD15*

DOI 10.1080/00365520310006612

haplotypes is lower in African-Americans with Crohn disease compared to Caucasian cohorts (16). In Japanese patients, mutations or SNPs do not seem to play an important role in the susceptibility to Crohn disease (17, 18). Since *NOD2/CARD15* mutants have been seen with different frequencies in geographically diverse populations and are an important contributory factor of Crohn disease (9–17, 19), investigation of the range of mutations of *NOD2/CARD15* for possible relations of genotype and variability of phenotypic presentation of Crohn disease seems worthwile (20). The present study examines the prevalence of mutations in the *NOD2/CARD15* gene in a cohort of well-characterized patients with Crohn disease from Madrid, Spain, and the genotype–phenotype correlations in the disease process.

Materials and Methods

Study population

We studied a cohort of 204 Caucasian unrelated consecutive patients with Crohn disease who were recruited in a Unit of Inflammatory Bowel Disease (IBD) from a single tertiary referral center in Madrid, Spain. Diagnosis of Crohn disease was based on standard clinical, radiologic, endoscopic, and histologic criteria (19). Phenotypic details were obtained by review of clinical charts and personal interview with the patients. The same clinical questionnaire was completed for each patient. This questionnaire included: date of birth, sex, familial IBD, age at diagnosis, duration of follow-up, smoking habits, history of surgery (tonsillectomy, appendectomy), definitions of the Vienna classification for age at diagnosis (A1, $<$40 years, A2, $\geq$40 years), disease location (L1, terminal ileum, L2, colon, L3, ileocolon, L4, upper gastrointestinal), and behavior (B1, non-stricturing non-penetrating, B2, stricturing, B3, penetrating), perianal disease defined as the presence of perianal abscess, fistulas and/or ulceration), extraintestinal clinical manifestations (articular, cutaneous, ocular, hepatic), and previous treatment as an indication of severity of disease (surgical intervention, corticosteroids, immunosuppressant agents, infliximab). All patient data were recorded by a gastroenterologist from the Unit of IBD (J.L.M.) who was blind from the genotype status of each patient.

A total of 104 normal healthy unrelated controls from the same geographic area were recruited from the blood-donor system at the hospital. A medical history and a laboratory investigation were obtained from every individual to exclude pre-existing disorders. The protocol was approved by the institutional review board and all patients and controls included in the study gave their written informed consent.

Genotyping

Amplification and specific discrimination of alleles in all blood samples was performed by two independent laboratories using different techniques, gel electrophoresis at Vrije Universiteit Medical Centre, Amsterdam, and fluorimetric detection at Hospital Clínico San Carlos, Madrid. In the case of discordant results, specific sequencing of DNA strands was performed.

Briefly, genotyping at Vrije Universiteit Medical Centre was performed as follows: *NOD2/CARD15* L1007fs. A frameshift mutation produced by a C-insertion in nucleotide 3020 of *NOD2/CARD15* gene affecting the 10th leucine rich-repeat region of the protein was amplified by multiple PCR as described previously (3).

NOD2/CARD15 G908R (G908R). The G→C substitution at position 2722 in the 8th exon of *NOD2/CARD15* gene (NCBI SNP Id rs 2066845) abolishes an *HhaI* site: 5′-AAGTCTGTAATGTAAAGCCAC- 3′ (sense) and 5′-CCC-AGCTCCTCCCTCTTC- 3′ (antisense). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 5 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s, and final incubation at 72 °C for 7 min. HhaI digestions of the PCR product resulted in fragments that either remained intact (*NOD2/CARD15* G908 allele) or were cut into two fragments (*NOD2/CARD15* 908R allele).

NOD2/CARD15 2104T gene polymorphism (R702W). A bi-allelic polymorphism produced for a C→T substitution at position 2104 in the 3rd intron of the *NOD2/CARD15* gene (NCBI SNP Id rs#2066844) produces the *Hpall* restriction site. This region was amplified by PCR with the following primers (Invitrogen Life Technologies, Breda, The Netherlands): 5′-CGCACAACCTTCAGATCACA- 3′ (sense) and 5′- GGATGGAGTGGAAGTGCTTG- 3′ (antisense). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 1 min. *HpaII* digestion of the PCR product fragments was cut into either three fragments (OPCIONAL of 64-bp, 54-bp and 47-bp) (*NOD2/CARD15* R702 allele) or two fragments (OPCIONAL of 114-bp and 47-bp, respectively) (*NOD2/CARD15* 702W allele).

Genotyping at Hospital Clínico San Carlos was performed as follows: The L1007fs was genotyped by use of the Taqman system (Applied Biosystems, Foster City, Calif., USA) with primers and probes that had been synthesized according to a previously described protocol (4). Taqman-PCR products were read directly into an ABI 7700 analyzer (Applied Biosystems).

The G908R was genotyped by specific amplification of the allele using the following primers: 5′-TTGCCTTTTCA-GATTCTGGG- 3′ (wild type, sense) and 5′-TTGCCTTTT-CAGATTCTGGC- 3′ (mutated, sense) and 5′-CCCCTC-GTCACCCACTCTG- 3′ as antisense primer in both cases. The PCR conditions were as follows: initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturing at 94 °C for 15 s and annealing at 65 °C for 30 s. Detection of the normal/mutant amplified product in an ABI 7700 analyzer was performed using the fluorescent stain SyberGreen

according to the manufacturer's instructions (Applied Biosystems).

Genotyping of the R702W was performed following the same method by substitution of C/T in exon 3/4 with primers 5′-CATCTGAGAAGGCCCTGTTC(C/T)-3′ and 5′-CAGACACCAGCGGGCACA- 3′ and amplification cycles at 94 °C for 15 s and at 65 °C for 30 s.

Statistical analysis

The frequencies for the *NOD2/CARD15* mutations were estimated by counting gene and calculating sample proportions. Carrier status was considered if any subject inherited at least one copy of the variant allele. Compound heterozygous status was defined as the presence of two different variants, and no variant. Case-control analyses were performed with the chi-squared statistics or Fisher exact test. The association between *NOD2/CARD15* mutations and phenotypic characteristics of Crohn disease was estimated by the odds ratio (OR) with the 95% confidence interval (CI). To assess whether *NOD2/CARD15* variants influence synergically upon the course of Crohn disease, subjects were classified as carriers and non-carriers of variant allele at both polymorphic loci. The chi-squared test or Fisher exact test was used for the comparison of carriers and non-carriers. When the association between genotype and phenotypic variables was known, statistical correction was not applied. Logistic regression analysis was perfomed to assess whether *NOD2/CARD15* mutations were correlated with a particular clinical phenotype. Association was expressed as relative risk (RR) with 95% CI. The multiple logistic regression analyses were adjusted for age (years). A two-tailed P value ≤ 0.05 was considered as significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 10.07 for Windows (SPSS Inc., Chicago, Ill., USA).

Results

The cohort of 204 patients with Crohn disease consisted of 108 men and 96 women. The median age at diagnosis was 27 years (mean 31.6, range 8–80) with an interquartile range of 22–26 years. The median duration of follow-up was 8 years (mean 9.9, range 0.6–44) with an interquartile range of 5–13 years.

At least one mutation was present in 32.8% of patients compared to 10.7% in controls (OR = 4.08, 95% CI 2.21 to 7.50, $P < 0.05$)). In patients with Crohn disease, the frequency of R702W, G908R, and L1007fs carriers was 13.7%, 8.3%, and 14.2%, respectively. Compound heterozygotes and homozygotes for a variant occurred in 3.4% and 2.9% of patients and in none of the controls (Table I).

The genotype–phenotype correlations are given in Table II. With regard to Vienna classification of the disease, stricturing behavior was significantly associated with G908R carriership ($P = 0.002$). Specifically, patients with stricturing disease had a 3.38 increased risk (95% CI, 1.86 to 6.28) of carrying the G908R allele variant. The presence of at least one mutant variant was positively associated with ileal disease ($P = 0.001$) and inversely associated with solely colonic involvement ($P = 0.007$), that is, patients with ileal disease had a 1.61 increased risk (95% CI, 1.21 to 2.15) of carrying at least one mutation. The risk ratio for solely colonic involvement was 0.29 (95% CI, 0.11 to 0.80). In the multivariate analysis, the L1007fs variant was mostly associated with ileal disease ($P = 0.0061$, RR = 3.10, 95% CI, 1.33 to 7.2). No other variables of the Vienna classification were associated with the presence or absence of *NOD2/CARD15* variants. On the other hand, in relation to risk factors for Crohn disease, a significant association of the G908R allele carriership and history of appendectomy was observed ($P = 0.02$, RR = 2.54, 95% CI, 1.22 to 5.30). Surgery over the course of the disease was significantly more frequent in patients who were carriers of G908R allele ($P = 0.005$, RR = 2.4, 95% CI, 1.64 to 3.51), but in the logistic regression analysis this variable was dependent not only on the presence of G908R mutation ($P = 0.028$, RR = 5.13, 95% CI, 1.65 to 15.88) but also of ileal disease ($P < 0.001$, RR = 3.25, 95% CI, 1.73 to 6.10), and stricturing disease behavior ($P < 0.001$, RR = 6.15, 95% CI, 1.03 to 22.10). No other risk factors, clinical manifestations, and treatment modalities were associated with *NOD2/CARD15* variants. A gene-dosage effect on phenotypic characteristics was not observed.

Discussion

We have performed a genotype-phenotype correlation in a cohort of 204 Caucasian patients with Crohn disease from the

Table I. Distribution of the CARD15 L1007fs, G908R and R702W carriership. Frequencies in Spanish patients with Crohn disease and controls

*NOD2/CARD1*5 carriership	Patients ($n = 204$)	Controls ($n = 104$)	Odds ratio (95% CI)	P value
R702W	28 (13.7)	6 (4.3)	3.55 (1.43–8.84)	0.0039
G908R	17 (8.3)	3 (2.1)	4.15 (1.19–14.45)	0.016
L1007fs	29 (14.2)	6 (4.3)	3.70 (1.49–9.17)	0.0028
At least one variant	67 (32.8)	15 (10.7)	4.08 (2.21–7.50)	<0.0001
Heterozygote	54 (26.5)	15 (10.7)	3.0 (1.61–5.57)	<0.0001
Compound heterozygote	7 (3.4)	0		
Homozygote	6 (2.9)	0		

Data as number and percentage in parentheses.

Table II. Genotype–phenotype relation in 204 patients with Crohn disease

Phenotypic characteristics	Total no. (%)	Carrier frequency, no. (%)				Mutant genotype, no. (%)		
		R702W	G908R	L1007fs	At least one mutant variant	Heterozygote	Compound heterozygote	Homozygote
Sex								
Men	108 (52.9)	10 (4.9)	6 (2.9)	16 (7.8)	28 (13.7)	20 (9.8)	4 (2.0)	4 (2.0)
Women	96 (47.1)	18 (8.8)	11 (5.4)	13 (6.4)	39 (19.1)	34 (16.7)	3 (1.5)	2 (1.0)
Age at diagnosis								
A1, <40 years	160 (78.4)	24 (11.8)	13 (6.4)	24 (11.8)	56 (27.0)	45 (22.1)	6 (2.9)	5 (2.4)
A2, ≥40 years	44 (21.6)	4 (2.0)	4 (2.0)	5 (2.4)	11 (5.4)	9 (4.4)	1 (0.5)	1 (0.5)
Family history	23 (11.3)	4 (2.0)	3 (1.5)	1 (0.5)	7 (3.4)	5 (2.4)	1 (0.5)	1 (0.5)
Smokers	110 (53.9)	15 (7.3)	8 (3.9)	11 (5.4)	35 (17.1)	28 (13.7)	3 (1.5)	4 (2.0)
Appendectomy	32 (15.7)	4 (2.0)	3 (1.5)	1 (0.5)	10 (4.9)	6 (2.9)	3 (1.5)	1 (0.5)
Tonsillectomy	28 (13.7)	3 (1.5)	2 (1.0)	2 (1.0)	7 (3.4)	7 (3.4)	0	1 (0.5)
Disease behavior								
B1, Non-stricturing, non-penetrating	83 (40.7)	10 (4.9)	4 (2.0)	9 (4.4)	22 (10.8)	20 (9.8)	1 (0.5)	1 (0.5)
B2, Stricturing	34 (16.7)	5 (2.4)	8 (3.9)	6 (2.9)	16 (7.8)	12 (5.9)	3 (1.5)	1 (0.5)
B3, Penetrating	87 (42.6)	13 (6.4)	5 (2.4)	14 (6.9)	29 (14.2)	22 (10.8)	3 (1.5)	4 (2.0)
Location of disease								
L1, terminal ileum	93 (45.6)	15 (7.3)	11 (5.4)	20 (9.8)	41 (20.1)	31 (15.2)	5 (2.4)	5 (2.4)
L2, colon	32 (15.7)	4 (2.0)	0	0	4 (2.0)	4 (2.0)	0	0
L3, ileocolon	71 (34.8)	8 (3.9)	5 (2.4)	9 (4.4)	20 (9.8)	17 (8.3)	2 (1.0)	1 (0.5)
L4, upper gastrointestinal	8 (3.9)	1 (0.5)	1 (0.5)	0	2 (1.0)	0	2 (1.0)	0
Perianal	46 (22.5)	5 (2.4)	1 (0.5)	5 (2.4)	11 (5.4)	10 (4.9)	0	1 (0.5)
Extraintestinal clinical manifestations								
Cutaneous	27 (13.2)	4 (2.0)	4 (2.0)	3 (1.5)	11 (5.4)	9 (4.4)	0	2 (1.0)
Articular	36 (17.6)	5 (2.4)	3 (1.5)	7 (3.4)	12 (5.9)	7 (3.4)	3 (1.5)	2 (1.0)
Treatment								
Surgical intervention	67 (32.8)	5 (2.4)	12 (5.9)	12 (5.9)	25 (12.2)	18 (8.8)	4 (2.0)	3 (1.5)
Infliximab	25 (12.3)	5 (2.4)	1 (0.5)	2 (1.0)	8 (3.9)	7 (3.4)	0	1 (0.5)
Immunosuppressants	63 (30.9)	10 (4.9)	6 (2.9)	8 (3.9)	24 (11.8)	23 (11.3)	0	1 (0.5)

Autonomous Community of Madrid (central Spain) who had been followed for a mean of 9.9 years. The clinical diagnosis of Crohn disease was confirmed by the criteria of Gasche et al. (8) Genotyping was performed at two independent laboratories and discordant cases were resolved by DNA specific sequencing. In this respect, the application of two different techniques for *NOD2/CARD15* genotyping of the three important SNPs supports the quality of the data despite the relatively small cohort.

The prevalence of carriership for *NOD2/CARD15* gene polymorphisms in the Spanish population with Crohn disease (R702W, 13.7%; G908R, 8.3%; L1007fs, 14.2%) is similar to that obtained in studies of Caucasian patients from central and northern Europe (9, 10, 12, 13, 17), Canada (14), and North America (3) and much higher than the prevalence of the three mutant variants found in healthy people from our same geographic area (R702W, 4.3%; G908R, 2.1%; L1007fs, 4.3%). Neither homozygosity nor compound heterozygosity for a variant was observed in controls, which confirms the strong genetic contribution of this gene to susceptibility for Crohn disease (2). However, a complex interplay of genetic and environmental factors is supported by patterns of geographic variation in the incidence of Crohn disease (e.g. three or more times higher in northern than in southern Europe) (22–24) and significant racial differences in the frequency of major mutations (R702W; G908R and L1007fs) in the *NOD2/CARD15* gene (16–19). On the other hand, the possibility of characterizing phenotypic subtypes in a heterogeneous disorder such as Crohn disease according to location and behavior of the disease may convert genotyping of candidate genes, including the *NOD2/CARD15* gene, to a promising tool for understanding disease development and progression (25).

Although in some studies mutations in the *NOD2/CARD15* gene are not a marker of Crohn disease according to the Vienna classification (26, 27), most reports describe an association between *NOD2/CARD15* polymorphisms and disease phenotype (10–15, 28). In our Spanish Crohn disease population and in agreement with findings in an Italian population (27), mutations of the *NOD2/CARD15* gene were not associated with age at diagnosis, which is in contrast to data in northern and central Europe (2, 10, 12, 28) and in the Ashkenazi Jewish population (29) showing that patients with mutations in the *NOD2/CARD15* gene presented earlier than patients with no *NOD2/CARD15* variants. This is consistent with the epidemiologic observation in northern and central Europe that Crohn disease is diagnosed at a younger age and is associated with a higher rate of complications compared to cases from sourthern Europe, which in turn stresses the importance of studies designed to examine gene–gene and gene–environmental interactions (30).

Location of disease is the variable that remains more stable during the course of the disease (31) and that showing a stronger association with mutations of the *NOD2/CARD15*

gene in genotype–phenotype studies using the Vienna classification. In our study, like others (10, 15, 28), possession of a *NOD2/CARD15* variant, particularly L1007fs, was significantly associated with ileal disease. On the other hand, mutations of the *NOD2/CARD15* gene were exceptional in patients with solely colonic involvement. This subgroup of patients accounted for 16% of the patients and showed a prevalence of the variant allele similar to that in health controls, which may indicate that in this subgroup of patients other genes may be involved in the susceptibility to Crohn disease (28). In contrast to findings in the Norwegian and German populations (12) and similarly to findings in the British population (13, 28), we have not found an association with location of disease in the right colon and mutations of the *NOD2/CARD15* gene.

In our population of patients with Crohn disease, an association between stricturing disease and carriers of the G908R polymorphism was observed, although stricturing behavior was mainly dependent on location of disease in the terminal ileum. In contrast to observations in other populations, especially from northern and central Europe (4, 12), no association of the L1007fs and R702W polymorphisms and a fibrostenotic behavior was found. In addition, and in contrast to other studies (10, 12, 14, 28), no other extraintestinal manifestations and perianal disease were associated with mutations of the *NOD2/CARD15* gene.

With regard to risk factors for Crohn disease, we only found a significant association between carriership of the G908R allele and history of appendectomy, which was independent of location and behavior of the disease. Russel et al. (32) also noted a positive association of Crohn disease and previous appendectomy, suggesting that appendectomy in some cases was a result of still undiagnosed Crohn disease.

Surgery over the course of the disease was significantly more frequent in patients who were carriers of the G908R allele, but this variable was dependent not only on the carriership of G908R allele but also of ileal disease and a stricturing disease behavior. It therefore seems that mutations of the *NOD2/CARD15* gene do not exert an isolated influence on the prognosis of Crohn disease. In other studies (33, 34), *NOD2/CARD15* was not predictive of treatment outcome with infliximab in patients with Crohn disease. Finally, no gene-dosage effect (homozygotes, compound heterozygotes) on phenotypic characteristics was observed. This could indicate that a higher genetic load would increase susceptibility to Crohn disease but would not affect the clinical characteristics of the disease. In contrast, in the study of Lesage et al. (10) patients with double-dose mutations were characterized by a younger age at onset, a more frequent stricturing phenotype, and a less frequent colonic involvement than was seen in those patients who had no mutation.

This study has shown that in a Spanish population from Madrid, mutations of the *NOD2/CARD15* gene were a marker of susceptibility for Crohn disease and were associated with ileal disease. Carriers of the G908R mutation were characterized by a stricturing disease behavior, history of appendectomy, and surgical interventions over the course of the disease. Identification of plausible factors that may interact with mutations in the *NOD2/CARD15* gene is a promising step toward understanding how sequence variation influences disease susceptibility in Crohn disease.

Acknowledgements

Laura S. Murillo is a fellow of the Prous Science Foundation, Barcelona, Spain. We thank Laboratorios Andromaco/Grunenthal, S.A., Madrid, Spain, for financial support of logistic aspects of the research and Marta Pulido, M.D., for editing the manuscript and editorial assistance. The study was also supported by grant 01/108-03 from Fondo de Investigación Sanitaria (FIS), Madrid, Spain. Juan L. Mendoza and Laura S. Murillo contributed equally to this article.

References

1. Shanahan F. Crohn's disease. Lancet 2002;359:60–2.
2. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599–603.
3. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease. Nature 2001;411:603–6.
4. Hampe J, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, et al. Association between insertion mutation in *NOD2* gene and Crohn's disease in German and British populations. Lancet 2001;357:1925–8.
5. Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. Gastroenterology 2003;124:993–1000.
6. Rosenstiel P, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D, et al. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. Gastroenterology 2003;124:1001–9.
7. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 2001;276:4812–8.
8. Gasche C, Scholmerich J, Brynskov J, D'Haens G, Hanauer SB, Irvine EJ, et al. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. Inflamm Bowel Dis 2000;6:8–15.
9. Ahmad T, Marshall SE, Mulcahy-Hawes K, Orchard T, Crawshaw J, Armuzzi A, et al. High resolution MIC genotyping: design and application to the investigation of inflammatory bowel disease susceptibility. Tissue Antigens 2002;60:164–79.
10. Lesage S, Zouali H, Cezard JP, Colombel JF, Belaiche J, Almer S, et al. *CARD15/NOD2* mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet 2002;70:845–7.
11. Radlmayr M, Torok HP, Martin K, Folwaczny C. The c-insertion mutation of the *NOD2* gene is associated with fistulizing and fibrostenotic phenotypes in Crohn's disease. Gastroenterology 2002;122:2091–2.
12. Hampe J, Grebe J, Nikolaus S, Solberg C, Croucher PJ, Mascheretti S, et al. Association of *NOD2* (*CARD 15*) genotype with clinical course of Crohn's disease: a cohort study. Lancet 2002;359:1661–5.
13. Cuthbert AP, Fisher SA, Mirza MM, King K, Hampe J, Croucher PJ, et al. The contribution of *NOD2* gene mutations to the risk

and site of disease in inflammatory bowel disease. Gastroenterology 2002;122:867–74.
14. Vermeire S, Wild G, Kocher K, Cousineau J, Dufresne L, Bitton A, et al. *CARD15* genetic variation in a Quebec population: prevalence, genotype–phenotype relationship, and haplotype structure. Am J Hum Genet 2002;71:74–83.
15. Vavassori P, Borgiani P, D'Apice MR, De Negris F, Del Vecchio Blanco G, Monteleone I, et al. 3020insC mutation within the *NOD2* gene in Crohn's disease: frequency and association with clinical pattern in an Italian population. Dig Liver Dis 2002;34: 153.
16. Bonen DK, Nicolae DL, Moran T, Turkyilmaz MA, Ramos R, Karaliukas R, et al. Racial differences in *NOD2* variation: characterization of *NOD2* in African-Americans with Crohn's disease. Gastroenterology 2002;122 Suppl:A29.
17. Inoue N, Tamura K, Kinouchi Y, Fukuda Y, Takahashi S, Ogura Y, et al. Lack of common *NOD2* variants in Japanese patients with Crohn's disease. Gastroenterology 2002;123:86–91.
18. Yamazaki K, Takazoe M, Tanaka T, Kazumori T, Nakamura Y. Absence of mutation in the *NOD2*/CARD15 gene among 483 Japanese patients with Crohn's disease. J Hum Genet 2002;47: 469–72.
19. Bonen DK, Ogura Y, Nicolae DL, Inohara N, Saab L, Tanabe T, et al. Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology 2003;124:140–6.
20. Farrokhyar F, Swarbrick ET, Irvine EJ. A critical review of epidemiological studies in inflammatory bowel disease. Scand J Gastroenterol 2001;36:2–15.
21. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol 1989;170 Suppl:2–6.
22. Loftus EV, Jr, Sandborn WJ. Epidemiology of inflammatory bowel disease. Gastroenterol Clin North Am 2002;31:1–20.
23. Shivananda S, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L, et al. Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). Gut 1996;39:690–7.
24. Crichton D, Arnott I, Watts D, Mowat C, Hutchinson J, Drummond H, et al. *NOD2/CARD15* mutations in a Scottish Crohn's disease population. Gastroenterology 2002;122 Suppl: A298.
25. Watts DA, Satsangi J. The genetic jigsaw of inflammtory bowel disease. Gut 2002;50 Suppl III:iii31–iii36.
26. Murillo L, Crusius JB, van Bodegraven AA, Alizadeh BZ, Peña AS. *CARD15* gene and the classification of Crohn's disease. Immunogenetics 2002;54:59–61.
27. Annese V, Latiano A, Andreoli A, Astegiano M, Bollani B, Campieri M, et al. Mutations of NOD_2 gene weakly correlate with the Vienna classification of Crohn's disease (SD). Gastroenterology 2002;122 Suppl:A–296.
28. Ahmad T, Armuzzi A, Bunce M, Mulcahy-Hawes K, Marshall SE, Orchard TR, et al. The molecular classification of the clinical manifestations of Crohn's disease. Gastroenterology 2002;122: 854–66.
29. Zhou Z, Lin XY, Akolkar P, Gulwani-Akolkar B, Levine J. Variation at NOD2 in familial and sporadic cases of Crohn's disease in the Ashkenazi Jewish population. Gastroenterology 2002;122 Suppl:A–296.
30. Lewis JD. The genesis of *IBD* genetics. Gastroenterology 2002; 123:2148–9.
31. Louis E, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi FA, Belaiche J. Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. Gut 2001;49:777–82.
32. Russel MG, Dorant E, Brummer RJ, van de Kruijs MA, Muris JW, Bergers JM, et al. Appendectomy and the risk of developing ulcerative colitis or Crohn's disease: results of a large case-control study. South Limburg Inflammatory Bowel Disease Study Group. Gastroenterology 1997;113:377–82.
33. Vermeire S, Louis E, Rutgeerts P, De Vos M, Van Gossum A, Belaiche J, et al. *NOD2/CARD15* does not influence response to infliximab in Crohn's disease. Gastroenterology 2002;123:106–11.
34. Mascheretti S, Hampe J, Croucher PJ, Nikolaus S, Andus T, Schubert S, et al. Response to infliximab treatment in Crohn's disease is not associated with mutations in the CARD15 (NOD2) gene: an analysis in 534 patients from two multicenter, prospective GCP-level trials. Pharmacogenetics 2002;12:509–15.

Received 24 June 2003
Accepted 19 September 2003