

The V249I polymorphism of the CX3CR1 gene is associated with fibrostenotic disease behavior in patients with Crohn's disease

Jean-Marc Sabate^a, Nejma Ameziiane^b, Jérôme Lamoril^b, Pauline Jouet^a, Jean-Pierre Farmachidi^a, Jean-Claude Soulé^c, Florence Harnois^a, Iradj Sobhani^c, Raymond Jian^d, Jean-Charles Deybach^b, Dominique de Prost^b and Benoit Coffin^a

Objectives CX3CR1, the receptor of CX3CL1/fractalkine, is involved in regulation of inflammatory response and the CX3CR1-I249-M280 naturally occurring mutants are associated with altered binding to the ligand. Our aim was to evaluate the frequency of CX3CR1 V249I and T280M polymorphisms and NOD2/CARD15 mutations in Crohn's disease patients and to search for a relationship with phenotype.

Methods Clinical data were retrospectively collected. V249I and T280M polymorphisms of CX3CR1 gene and NOD2/CARD15 mutations (R702W, G908R, 3020InsC) were identified.

Results Two hundred and thirty-nine patients (140 females, 39.7 ± 14.1 years) were included. About 37.4% were heterozygous and 8.8% were homozygous for the V249I CX3CR1 polymorphism, 18.1% were heterozygous and 1.3% homozygous for the T280M CX3CR1 polymorphism and 35.9% had at least one of the three mutations of NOD2/CARD15. The T280M CX3CR1 polymorphism was not associated with any phenotype. In univariate analysis, stenosis was significantly associated with both V249I CX3CR1 polymorphism and 3020InsC NOD2/CARD15 mutations. In smoker patients carrying the CX3CR1 allele I249, there was a significant increase in the frequency of fibrostenosing disease [$P=0.005$, odds ratio (OR): 3.25] whereas this relationship disappeared in the

group of nonsmokers ($P=0.72$). In multivariate analysis, 3020InsC NOD2/CARD15 mutations and the V249I CX3CR1 polymorphism were independent risk factors for intestinal stenosis ($P=0.046$, OR: 1.8 and $P=0.044$, OR: 2.4, respectively).

Conclusion In Crohn's disease, V249I CX3CR1 polymorphism is associated with intestinal strictures, particularly in smokers. This association is independent of CARD15 mutations. *Eur J Gastroenterol Hepatol* 20:748–755 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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^aDepartment of Gastroenterology and Hepatology, ^bFédération de Génétique Moléculaire, AP-HP, Louis Mourier Hospital, Colombes, ^cDepartment of Gastroenterology and Hepatology, AP-HP, Bichat Hospital and ^dDepartment of Gastroenterology and Hepatology, AP-HP, European Georges Pompidou Hospital, Paris, France

Correspondence to Benoit Coffin, AP-HP, Assistance Publique-Hôpitaux de Paris, Service d'Hépatogastroentérologie, Hôpital Louis Mourier, 178 rue des Renouillers, Colombes 92700, France
Tel: +33 1 47 60 60 61; fax: +33 1 47 60 60 72;
e-mail: benoit.coffin@lmr.aphp.fr

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Introduction

Crohn's disease (CD) is a chronic, heterogeneous transmural inflammatory bowel disease (IBD) characterized by a dysregulated mucosal immune response. Epidemiological and linkage studies suggest that genetic factors play a significant role in determining susceptibility but environmental factors may also contribute to the pathogenesis of CD. Recently, coding region polymorphisms in the NOD2/CARD15 gene have been established to increase susceptibility to CD [1,2] and appear to be an important determinant of CD phenotype expression. Carriage of one or more of these mutations has been reported to be associated with earlier onset of CD [3–5],

ileal disease location [3–8] and, less consistently, fibrostenotic disease [3,8,9].

Functional studies have demonstrated that mutations within the NOD2/CARD15 gene contribute to CD susceptibility by inducing inappropriate responses to bacterial components which may alter signaling pathways in the innate immune system [10]. This has highlighted the role of altered response to bacteria or bacterial antigens in CD patients [11]. NOD2/CARD15 is located intracellularly, recognizes muramyl dipeptide and mediates the interaction between the innate immune response and the host [12]. Bacteria can penetrate the

mucosal barrier directly and stimulate the cells of the mucosal immune system, namely monocytes, macrophages and dendritic cells, which synthesize inflammatory cytokines and chemokines, resulting in extensive tissue damage induced by activated leukocytes [13].

Chemokines, together with adhesion molecules, regulate the appropriate addressing and delivery of leukocytes and have been identified recently as regulators of intestinal epithelial cells [14]. More than 40 chemokines have been identified and are subdivided into four families: C, CC, CXC, and CX3C chemokines [15]. CX3CL1, also called fractalkine, is the only CX3C-chemokine [16]. CX3CL1 is expressed on the surface of many cell types, including interleukin-1 and tumor necrosis factor-activated endothelial and dendritic cells [17,18]. Interestingly, endothelial cells and epithelial cells of the intestinal mucosa also express CX3CL1 [19–21]. The CX3CL1 receptor, CX3CR1, is expressed on monocytes, natural killer (NK) cells, and lymphocytes [22], including intestinal intraepithelial lymphocytes [21], on neurons, microglial [19], and dendritic cells, where it controls the adherence of enteroinvasive pathogens at this level [18]. After stimulation by bacteria or bacterial product degradation, CX3CR1-expressing cells rapidly adhere to the inflamed vascular endothelium and may play a role as a vascular gateway for cytotoxic effector cells [23]. Two CX3CR1 polymorphisms, V249I and T280M, that are in complete linkage disequilibrium, are associated with interindividual differences in susceptibility to HIV infection [24] and to a decreased risk of coronary artery disease [17,25]. In patients with rheumatoid arthritis, a study suggested that fractalkine may represent a Th1-type chemokine with a proinflammatory role, and a trend toward increased severity of disease was found in patients carrying CX3CR1 variants [26,27]. Like the NOD2/CARD15 gene, the fractalkine gene is located on the chromosome 16q [28]. Recent data showed that the mucosal chemokine response was upregulated during inflammatory bowel disease (IBD) [29] and from a theoretical point of view, fractalkine could be involved in the pathophysiology of certain phenotypes as it may act as a chemoattractant or an adhesive protein.

The aims of this study were to characterize the frequency of NOD2/CARD15 mutations and CX3CR1 polymorphisms in a retrospective cohort of CD patients, and to search for a relationship between genotype and phenotype.

Patients and methods

Patient population

Between February 2001 and October 2002, every consecutive patient with a known diagnosis of CD who came to three different academic gastroenterology units of the Assistance Publique-Hôpitaux de Paris (Hôpital

Louis Mourier, Hôpital Bichat and Hôpital Européen Georges Pompidou) was included in the study. Diagnosis of CD was based on established clinical, endoscopic, radiological, and histological criteria according to the usual French criteria [30]. Age at diagnosis, topography of lesions at onset, disease behavior at latest follow-up, surgical resection history, tobacco use, oral contraceptive use, extraintestinal manifestations, family history, and presence of granuloma based on biopsy specimens were collected retrospectively by four investigators blinded to genotype. The new validated Montreal classification was also used to describe patients' characteristics [31].

Each patient gave a written informed consent to the protocol that was approved by the Ethics Committee of Saint-Germain en Laye Hospital (France).

Disease site designation

Disease location was classified as small bowel (every lesion located between the oesophagus and the ileocecal valve, using endoscopy, small bowel series), colon (every lesion located between the ileocecal valve and the anus) or small bowel and colon (association of both types of lesions). Disease behavior was also noted, with the mention of fibrostenosing or fistulating disease. Patients were considered to have fibrostenosing disease if they had documented persistent occlusive syndrome, required endoscopic dilatation and/or surgery (resection or strictureplasty) for intestinal obstruction. Fistulating disease was defined by internal or perianal perforating disease. Perianal disease was recorded if patients had current or previous evidence of either perianal fistulae or abscesses or rectovaginal fistulae.

Control group

A control group of 216 patients with no clinical history of CD was used to characterize the frequency of NOD2/CARD15 polymorphisms and V249I and T280M CX3CR1 polymorphisms in the general population.

Genotyping

Screening for the R702W, G908R, and 3020InsC polymorphisms in the NOD2/CARD15 gene

Genomic DNA was prepared from peripheral blood cells by using standard procedures and was stored at -20°C until analysis. The three CARD15 sequences were amplified using primers designed by Proligo (Paris, France) (Table 1). PCRs were performed using a 50- μl reaction mixture containing: 10 pmol of each primer, 200 $\mu\text{mol/l}$ of each dNTP, $1 \times$ PCR buffer, 2 mmol/l MgCl_2 , 1 U of *Taq* DNA polymerase (Sigma, Saint Quentin Fallavier, France) and 2 μl (100 ng) of DNA. The PCR program was as follows: an initial denaturation step was performed at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 s, reannealing at the temperatures given in Table 1 for 30 s and extension at 72°C for 30 s. The PCR was ended by a final extension at

Table 1 Primers, probes and enzymes used for genotyping the NOD2/CARD15 and CX3CR1 polymorphisms

Designation	Primers and probes (5'-3')	Fragment size (bp)	Enzymes	Annealing temperature (°C)
CARD15 P268S	F: CTCAGTCTCGCTTCTCAGT R: ACTCGGTGCGGATGTACTTC	630	<i>DpnII</i>	58
CARD15 R702W	F: GCCGAGCCGCACAACCTTCA R: GCACCAGACCCAGCACATAG	227	<i>HpaII</i>	52
CARD15 G908R	F: GATTGAGTGGTCTGCCCT R: TACTCCATTGCCTAACATTGTG	324	<i>HhaI</i>	56
CARD15 3020InsC	F: CTGAGCCTTTGTTGATGAGC R: TCTTCAACCACATCCCCATT	533	<i>NlaIV</i>	54
CX3CR1 T280M	F: TTGTGACATGAGGAAGGATCTGAG R: AGGTGGTAAAGGTATCTTCTGAAC P1: FAM-ACTG ^L A ^L GA ^L CG ^L GT ^L TGCAT-TAMRA P2: JOE-TGACTG ^L A ^L GA ^L TG ^L GT ^L TGCAT-TAMRA	129		64
CX3CR1 V249I	F: AGCCAAAGCCATTAACT R: CCAGCCTCAGATCCTTC P3: FAM-AAA ^L AT ^L C ^L AT ^L A ^L AC ^L GT ^L TG-TAMRA P4: JOE-GAAA ^L A ^L T ^L C ^L AT ^L A ^L AT ^L GT ^L TG-TAMRA	143		64

F, forward primer; FAM, 6-carboxyfluorescein; JOE, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein; ^L, locked nucleic acid (LNA) base; P1, T280M wild-type probe; P2, T280M mutated probe; P3, V249I wild-type probe; P4, V249I mutated probe; R, reverse primer; TAMRA, 6-carboxytetramethylrhodamine.

72°C for 10 min. The PCR products were digested overnight at 37°C by the enzymes shown in Table 1, and checked on 3% agarose gels.

Screening for V249I and T280M polymorphisms in the CX3CR1 gene

V249I and T280M genotyping were performed using a modified 5'-nuclease assay and locked nucleic acid probes [32]. All PCRs were performed in a final volume of 25 µl. The final concentration of the primers and probes listed in Table 1 were 300 and 100 nmol/l, respectively. Two microliters of template DNA (50–500 ng) and 12.5 µl of 2x No ROX TaqMan Universal PCR Master Mix (Eurogentec, Seraing, Belgium) were used for the assay. The final concentration of MgCl₂ in the mixture was adjusted to 5 mmol/l. PCR conditions were as follows: 2 min at 50°C to activate uracil-*N*-glycosylase, 10 min at 95°C to activate the hot-start DNA polymerase and deactivate uracil-*N*-glycosylase, followed by 45 cycles of 15 s at 95°C and 1 min at 64°C.

Statistical analysis

Results were expressed as means and standard deviation or medians and interquartile range if a non-Gaussian distribution was observed. Frequencies of NOD2/CARD15 and CX3CR1 were compared by means of the χ^2 test or Fisher's exact test when appropriate, to evaluate the association between carriers and noncarriers of the different alleles or between genotypes and categorical variables such as disease location and behavior. Multivariate analysis was performed with the logistic regression model to test the association between genotypes and phenotypic variables that were significantly associated with the allele variant from the

univariate analyses, adding variables according to the literature results and variables which showed a trend for statistical significance in univariate analysis in our series ($P < 0.10$). V249I and T280M were forced into the model, other additional predictors were selected using forward selection based on the Wald test. The linear regression model was used to search for a relationship between age at diagnosis and other variables. The stratified association test was also used to show whether the association between allelic variant and a phenotype (e.g. fibrostenosing disease) was primary or secondary to other variables (e.g. smoking). Analysis was performed with SPSS computer software (version 11.5). A P value of less than 0.05 was considered as statistically significant.

Results

Two hundred and thirty-nine CD patients with complete clinical information were analyzed from the three centers. Demographics and clinical characteristics of the population are presented in Table 2. Two hundred and thirty patients (96.6%) were Caucasian [including seven (2.9%) Jews], five Africans (2.1%), and four (1.7%) Orientals. Two-thirds of the patients had a follow-up of more than 5 years after the diagnosis of CD.

Surgery and medication history

Eighty-nine patients (37.2%) had a previous surgery for CD, with intestinal resection in 85 patients and/or stricturoplasty in 27 patients (11.3%). At the time of collecting clinical data, 35% of the patients were taking 5-aminosalicylic acid, 24.4% steroids, 15% budesonide, 50.2% azathioprine, 10.8% methotrexate, 0.5% ciclosporin, and 6.6% infliximab. Registration of past medication related to CD demonstrated that 91.6% of the patients

Table 2 Demographics and clinical characteristics of the study population (N=239)

Clinical characteristics	N=239
Sex (male/female)	99/140
Age (year)	39.7 ± 14.1
Disease duration (years)	9.3 ± 7.7
Familial history of IBD in first- or second-degree relatives (%)	13.6
Smoking habit at the time of the study (%)	
Never	48.6
Ex	17.9
Current	33.5
Oral contraception (% of women)	47.8
Disease location (%)	
Small bowel only	21.4
Colon only	30.8
Small bowel and colon	47.9
Perianal	38.9
Disease behavior (%)	
Stenotic disease	36.4
Fistulating disease	17.6
Extraintestinal manifestations (%)	31.5
Presence of granuloma (%)	35.8

IBD, inflammatory bowel disease.

received 5-aminosalicylic acid, 90.1% steroids, 33.8% budesonide, 71.4% azathioprine, 21% methotrexate, 4.7% ciclosporin, and 15.5% infliximab.

Age at onset and phenotypes

Mean age at diagnosis was 30.3 ± 12.7 years. According to the Montreal classification [31], the distribution of patients was as follows: age at diagnosis below 16 years (A1, $N=8.8\%$), between 17 and 40 years (A2, $N=71.4\%$), above 40 years (A3, $N=19.7\%$); disease location was ileal (L1, $N=17.6\%$), ileal + upper gastrointestinal (L1 + L4, $N=3.7\%$), colonic (L2, $N=28.2\%$), colonic + upper gastrointestinal (L2 + L4, $N=2.5\%$), ileocolonic (L3, $N=40.8\%$), ileocolonic + upper gastrointestinal (L3 + L4, $N=7.1\%$); disease behavior was non-stricturing nonpenetrating (B1, $N=46\%$), stricturing (B2, $N=36.4\%$), penetrating (B3, $N=17.6\%$), and perianal disease ($N=38.9\%$). Younger age at diagnosis was evidenced in patients with current or past small bowel involvement (28.3 ± 0.9 vs. 35.3 ± 1.6 years, $P < 0.001$) and in active smokers at the time of diagnosis (28.4 ± 1.0 vs. 32.0 ± 1.2 years, $P = 0.025$). No significant association was found between age at diagnosis and perianal disease ($P = 0.163$), fibrostenosing disease ($P = 0.084$) or a familial history of CD ($P = 0.305$).

Frequencies of genotypes

The genotypic frequencies of the NOD2/CARD15 and CX3CR1 polymorphisms in CD patients are shown in Table 3. Eighty-five CD patients (35.9%) and 36 (16.7%) control participants were carriers of at least one allelic variant of NOD2/CARD15 ($P < 0.001$). One hundred and nine CD patients (46.2%) and 108 (51.7%) control participants were carriers of either the I249 or M280 allele of CX3CR1 ($P = 0.25$). The frequencies for the

I249 or M280 alleles in patients and control groups were in accordance with the Hardy–Weinberg law.

In CD patients, there was no statistical difference in CX3CR1 genotype frequency between carriers and noncarriers of any allelic variant of NOD2/CARD15 (M280: 15.7 vs. 21.7%, respectively, $P = 0.264$; I249: 47.6 vs. 44.7%, respectively, $P = 0.67$).

Association between genotypes and phenotypes

The variant genotype frequencies of NOD2/CARD15 and CX3CR1 according to the presence or absence of small bowel only involvement, perianal disease and fibrostenosing disease are shown in Table 4. Presence of any NOD2/CARD15 mutation, was associated with younger age at diagnosis (28.3 ± 1.1 vs. 31.5 ± 1.1 years, $P = 0.042$), small bowel involvement ($P = 0.035$) and fibrostenosing behavior ($P = 0.0013$). In univariate analysis, the 3020InsC mutated genotype was significantly more frequent in patients with small bowel only involvement ($P = 0.037$), perianal disease ($P = 0.036$), and fibrostenosing behavior ($P = 0.001$). A familial history of CD was significantly more frequent in patients with 3020InsC allelic variants (30.4 vs. 10.9%, $P = 0.02$, respectively), as well as a carriage of any allelic variant of NOD2/CARD15 (56.5 vs. 33.6%, $P = 0.03$). Colonic disease, small bowel and colonic disease, and extraintestinal manifestations were not significantly more frequent in patients with NOD2/CARD15 allelic variants than in noncarriers (results not shown).

The I249 CX3CR1 allele was significantly more frequent in patients with fibrostenosing disease ($P = 0.035$) and less frequent in those with perianal disease ($P = 0.045$). This polymorphism was not associated with colonic disease, small bowel and colonic disease, small bowel only involvement, extraintestinal manifestations, and younger age at diagnosis. The duration of follow-up since diagnosis was longer in patients with intestinal stenosis than without intestinal stenosis (12.1 ± 7.7 vs. 7.8 ± 7.3 years, $P < 0.001$). Patients with or without V249I CX3CR1 polymorphism had the same duration of follow-up (9.5 ± 7.7 vs. 9.2 ± 7.8 years, respectively, $P = 0.72$). T280M CX3CR1 polymorphism was not significantly associated with age at diagnosis, topography of the disease, perianal disease, presence of fistulas, fibrostenosing disease, presence of familial history of CD, or extraintestinal manifestations.

None of the allelic variants tested was significantly associated with the presence of granuloma.

Environmental factors

Oral contraceptive

Among the 140 women, 47.8% used oral contraception at the time of data collection. No significant relationship

Table 3 Genotypic frequencies of NOD2/CARD15 and CX3CR1 polymorphisms in patients with CD (*N*=239) and controls (*N*=216)

Gene	Genetic variation		Patients		Controls		<i>P</i>
			(Present/tested)	%	(Present/tested)	%	
NOD2/CARD15	R702W	RR	192/237	81.0	190/215	88.4	0.042
		RW	45/237	19.0	25/215	11.6	0.042
		WW	0/237	0.0	0/215	0.0	1.00
	G908R	GG	221/237	93.2	211/215	98.1	0.02
		GR	14/237	5.9	4/215	1.9	0.05
		RR	2/237	0.8	0/215	0.0	0.52
	3020InsC	NN	207/237	87.3	196/215	91.2	0.25
		NI	25/237	10.5	15/215	7.0	0.24
		II	5/237	2.2	4/215	1.9	0.88
CX3CR1	V249I	VV	128/238	53.8	108/216	50.0	0.47
		VI	89/238	37.4	89/216	41.2	0.46
		II	21/238	8.8	19/216	8.8	0.87
	T280M	TT	191/237	80.6	154/216	71.3	0.027
		TM	43/237	18.1	57/216	26.4	0.045
		MM	3/237	1.3	5/216	2.3	0.62

3020InsC genotypes.

CD, Crohn's disease; I, insertion of a cytosine; N, no cytosine insertion.

Table 4 Relationship between NOD2/CARD15 and CX3CR1 genotypes and clinical phenotypes of CD cohort (*N*=239)

Clinical phenotype	R702W	G908R	3020InsC	Any NOD2 variant	V249I	T280M
Small bowel only						
Yes (%)	19.2	7.2	15.6	40.1	47.6	18.6
No (%)	18.6	5.7	5.7	25.7	42.9	21.4
<i>P</i>	0.916	0.680	0.037	0.035	0.502	0.611
Perianal disease						
Yes (%)	21.5	6.5	18.3	40.9	38	17.4
No (%)	17.4	6.9	9.0	32.6	51.4	20.7
<i>P</i>	0.427	0.883	0.036	0.198	0.045	0.532
Fibrotic stenosing						
Yes (%)	19.5	8.0	23	46.0	55.2	22.1
No (%)	18.7	6.0	6.7	30.0	41.1	17.9
<i>P</i>	0.869	0.545	0.001	0.013	0.035	0.430

could be evidenced between oral contraception and a specific phenotype.

Smoking

Overall smoking habits at the time of the study are shown on Table 2. At the time of CD diagnosis, the percentage of active smokers was higher (42.9%); between the date of diagnosis and the time of the study 29 patients stopped smoking and seven patients began to smoke. In active smokers at the time of the study, the mean number of cigarettes per day was 15.2 ± 7.7 and the mean duration of smoking habits was 16.0 ± 9.0 years, with a mean cumulative intake of 12.0 ± 8.0 cigarette pack years. A significant relationship was seen between disease location and tobacco use, as small bowel only involvement was more frequent in active smokers (85.3%) than in nonsmokers (14.7%) ($P = 0.001$; odds ratio: 3.14; 95% confidence interval 1.53–6.5). Patients with stenosis tended to be more frequently smokers (50.6 vs. 38.5%; $P = 0.075$). At the time of the study, among the patients with NOD2/CARD15 mutations, 53.8% were active or past smokers and 46.2% had never smoked ($P = 0.52$), and among the patients with V249I CX3CR1 polymorphism 51% were active or past smokers and 49% had never smoked ($P = 0.98$). Smoking was not significantly related

to specific phenotypes in patients with NOD2/CARD15 mutations. In smoker patients carrying CX3CR1 allele I249 but not in those carrying allele M280, there was a significant increase in the frequency of fibrotic stenosing disease ($P = 0.005$), as shown on Table 5, with a 3.25 times increase of risk, whereas this relationship disappeared in the group of nonsmokers ($P = 0.72$).

Multivariate analysis

The results of multivariate analysis in the cohort of CD patients for different clinical phenotypes are shown in Table 6. Binary logistic regression showed that fibrotic stenosing disease was independently associated with the V249I CX3CR1 polymorphism ($P = 0.046$), 3020InsC ($P = 0.044$) NOD2/CARD15 carriage, and smoking status ($P = 0.037$). Small bowel only involvement was associated with age at the time of diagnosis ($P < 0.001$). Perianal disease was significantly associated with 3020InsC variant genotypes ($P = 0.034$).

Multiple linear regression for age at the time of diagnosis showed that only smoking status was explicative ($P = 0.043$); neither the presence of at least one mutation of NOD2/CARD15 nor familial history of IBD was explicative.

Table 5 Relationship between V249I genotype frequencies of CX3CR1 and fibrostenotic phenotypes of CD cohort with stratification on smoking status (information present for 225 patients among 239)

Smoking status			Disease behavior		<i>P</i>	Odds ratio	95% CI
			No stenosis (<i>N</i> =142)	Stenosis (<i>N</i> =83)			
Nonsmoker (<i>N</i> =129)	V249I substitution	No	54.5% (48/88)	51.2% (21/41)	0.72	1.14	0.54–2.41
		Yes	45.5% (40/88)	48.8% (20/41)			
Smoker (<i>N</i> =96)	V249I substitution	No	66.7% (36/54)	38.1% (16/42)	0.005	3.25	1.40–7.53
		Yes	33.3% (18/54)	61.9% (26/42)			

CD, Crohn's disease; CI, confidence interval.

Table 6 Multivariate analysis in the cohort of 239 CD patients for different clinical phenotypes

	<i>P</i>	Odds ratio	95% CI
Fibrostenosing disease			
V249I	0.046	1.808	1.012–3.229
3020InsC	0.044	2.481	1.024–6.013
Smoker	0.037	1.863	1.038–3.342
Small bowel involvement			
3020InsC	0.111	2.538	0.807–7.988
Age at diagnosis	<0.001	0.960	0.939–0.982
Perianal disease			
3020InsC	0.034	0.429	0.196–0.940
V249I	0.054	1.699	0.991–2.912

Logistic regression analysis was performed for the genotypes that demonstrated an association by univariate analysis, adding variables according to the literature results and also variables that showed a trend for statistical significance in univariate analysis in our series.

CD, Crohn's disease; CI, confidence interval.

Discussion

In this study, we confirmed that NOD2/CARD15 mutations were significantly associated with small bowel location and fibrostenotic disease and demonstrated that the V249I CX3CR1 polymorphism was associated with fibrostenotic disease.

We found that younger age at diagnosis was associated with small bowel localization and tobacco use independently from the different mutations. Younger age at onset and family history of CD have been both reported to be associated with ileal rather than colon-only CD [33,34]. A trend toward a significant association between age at onset and smoking has been reported previously but this might be related only to the fact that smoking is more prevalent in younger patients [35].

In our population, the frequency of the three allelic variants of NOD2/CARD 15 was in the higher range of those reported previously in several cohorts of Western patients with CD [1,4,5,7,8,36–40] with R702W mutation in 19% of patients, the G908R in 6.7% and the 3020InsC in 12.7%. As reported previously by others, in univariate analysis, we found that 3020InsC allelic variant was significantly associated with small bowel involvement and fibrostenosing disease, whereas in multivariate analysis only fibrostenosing disease was associated with this allelic variant [3,5,8,9]. An association between NOD2/CARD15

and perianal location was specifically tested during four studies [3–5,9] but could not be evidenced. By contrast, we found a significant association between the 3020InsC allelic variant and perianal location, which remained significant after multivariate analysis. The high frequency of perianal location observed in our population, 38.9%, higher than in these studies [3–5,9] might be an explanatory factor. Finally, we could not find any significant relationship between NOD2/CARD15 mutations and the presence of granulomas on pathological examination. This point remains controversial as initial and more recent studies did not find a correlation between granulomas, detected on biopsies or surgical specimens [3,41] whereas Heresbach *et al.* [42] found a significant relationship between R702W mutation and the presence of granulomas.

Finally, we tested the hypothesis that the CX3CR1 polymorphism could be involved in some phenotypic expression in CD. We found 37.4% of heterozygous and 8.8% of homozygous carriers for the V249I polymorphism and 18.1% of heterozygous and 1.3% of homozygous carriers for the T280M polymorphism. Similar frequencies of heterozygous and homozygous carriers of the two CX3CR1 polymorphisms were recently found by Brand *et al.* [43] in a series of 206 CD patients. Looking at genotype–phenotype correlations, however, we found that the V249I polymorphism was significantly more frequent in patients with fibrostenotic disease and this association was independent of NOD2/CARD15 mutations. Intestinal stenosis often occurs during long follow-up, but duration of follow-up was similar in our study in patients with or without V249I CX3CR1 polymorphism. In Brand *et al.* [43], in univariate analysis, there was also a trend for more frequent V249I polymorphism in patients with intestinal stenosis (*P*=0.06). Additionally, they found that the T280 polymorphism was associated with ileocolon or ileal involvement and that homozygous carriers all had intestinal stenosis. In our study, only three patients were homozygous for T280M, this number is really small to come to a conclusion compared with the 16 patients in the German study, but two of these three patients had stenosis. As in the Brand *et al.* study [43], Bonferonni adjustments were not made because it was an explorative study, designed to test a specific hypothesis

about CX3CR1 polymorphisms and phenotype in CD. The other tests (CARD15 and phenotype) were performed to allow comparison with other series in the literature. For methodological rigor, the use of Bonferroni adjustments for multiple tests is mandatory but this has been discussed [44]. Applying this adjustment in our study, the *P* value for statistical significance in univariate analysis is lowered to 0.0083 and the association between V249I and fibrostenosing behavior is no more significant and becomes only a trend. However, we do not think that the association with CX3CR1 polymorphism is observed by chance, because of the Brand *et al.* study [43] and experimental data in animal models and in patients with CD that will be discussed in the following paragraph.

In our study, as in that of Brand *et al.* [43], the frequency of the two CX3CR1 polymorphisms was not higher in CD than in the control group and was similar to that reported previously in different populations [25,45] suggesting that it was not a key factor in the occurrence of CD. Functional data, however, suggest a role of CX3CR1 and fractalkine in CD. Immunohistochemistry demonstrated fractalkine expression in intestinal epithelial cell in normal small intestine and in active CD mucosae [21]. Brand *et al.* [43] demonstrated that fractalkine mRNA expression was significantly upregulated in intestinal epithelial cell lines after stimulation with proinflammatory cytokines such as TNF- α and IL-1 β , and was correlated with the level of inflammation *in vivo* in patients with CD. The expression of fractalkine is highest in the murine ileum compared with other segments, and CX3CR1 plays a role in bacterial clearance from the intestinal lumen by dendritic cells [18]. Daoudi *et al.* [46] showed that the V249I-T280M amino acid substitutions led to decreased adhesion to membrane-bound fractalkine. Recently, Sans *et al.* [47] have shown that production of fractalkine by microvascular cells is higher in IBD mucosa than in normal mucosa, and that greater numbers of T cells express CX3CR1 in the circulation of active IBD patients than in inactive IBD or in healthy participants.

In addition, stenosis was significantly more frequent in active smokers carrying the V249I CX3CR1 polymorphism. Smoking is a key environmental factor during CD [48]. Smoker CD patients had stenosis more frequently [35,49] and had a more frequent relapse of obstructive symptoms after endoscopic dilatation for stenosis than nonsmoker CD patients [50]. Smoking did not seem to increase the risk associated with NOD2/CARD15 mutations [5,8]. The relationship between tobacco and disease behavior remains largely unexplained but epidemiological data suggested it could be genetically supported. In homozygotic twins, smoking pairs had more risk of developing CD than ulcerative colitis and the frequency of smokers was increased among twins with CD fibrostenosing behavior [51]. The interaction between

tobacco and an inflammatory response to gut bacteria also warrants further studies [48]. Recently, it was demonstrated that exposure to tobacco can cause a decrease in dendritic cells and suppression of dendritic cell function [52,53]. If the link that we pointed out is confirmed, it could suggest that, as in other diseases [54], in CD a key environmental factor influencing disease behavior (tobacco) could be supported by a genetic susceptibility.

In conclusion, in this study, we confirmed the genotype-phenotype relationship in CD patients carrying NOD2/CARD15 mutations and we demonstrated that the V249I CX3CR1 polymorphism is probably not a predisposing factor to CD by itself, but that it was significantly associated to fibrostenotic disease behavior with a possible link with smoking.

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