IL23R, NOD2/CARD15, ATG16L1 and *PHOX2B* polymorphisms in a group of patients with Crohn's disease and correlation with sub-phenotypes

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Abstract. Recent genomic research has identified interleukin-23 receptor (*IL23R*), nucleotide-binding oligomerization domain containing 2 caspase-activation recruitment domain 15 (*NOD2/CARD15*), autophagy related 16-like 1 (*ATG16L1*) and paired-like homeobox 2b (*PHOX2B*) as susceptibility loci for Crohn's Disease (CD). Our aim was to investigate these gene variants in a group of CD patients and to analyse the correlation to sub-phenotypes such as gender, smoking habits, disease behaviour at diagnosis, severity of disease and extra-intestinal manifestations. Nineteen patients with CD and 20 healthy controls were included in the study. The gene variants IL23R rs7517847 and rs11209026, *NOD2/CARD15* rs2066845, *PHOX2B* rs16853571, *ATG16L1* rs2241879 and rs2241880 were genotyped by PCR followed by sequencing. The frequency of the G risk allele of *IL23R* rs7517847 was found to be increased in patients with CD (42%) compared to that in control subjects (20%) [odds ratio (OR), 2.9; 95% confidence interval [CI], 1.06-7.9; P=0.03]. In addition, the homozygous condition GG was also associated with CD (OR, 8.70; 95% CI, 0.9-81.6; P=0.038). The analysis of correlation of genotype to sub-phenotypes showed an association of *ATG16L1* rs2241879 with the lack of extra-intestinal manifestations (OR, 0.03; 95% CI, 0.002-0.45; P=0.006), and the patients defined as non-smokers displayed an increased frequency of the risk allele C (P=0.03). The present study confirms the association of the heterozygous and homozygous *IL23R* rs7517847 variant with CD and suggests an additive effect of smoking to the *ATG16L1* rs2241879 C risk allele SNP, in the context of the multifactorial model established for the development of CD and a protective effect of the same allele against extra-intestinal manifestations.

Introduction

Crohn's disease (CD) is a chronic inflammatory disease of the gastrointestinal tract. CD is characterised by persistent inflammation, often granulomatous, affecting all layers of the bowel wall. CD inflammation can occur anywhere from the mouth to the anus. Whereas in adults the disease is often limited to the ileum and/or colon, it is typically more extensive in children (1). The molecular basis of the pathogenesis of CD is not completely understood, but contributing factors may include persistent bacterial infections, a defective mucosal barrier, and an imbalance in the regulation of the intestinal immune response (2). CD is considered a complex disease with contributions from genetic, environmental and immunological factors. Strong epidemiological evidence for a genetic predisposition has stimulated recent efforts to identify the susceptibility genes (3,4).

In a genome-wide association analysis, the coding variants rs7517847 and rs11209026 of *IL23R*, involved in Th17-lymphocyte differentiation, were shown to be associated with CD. The intronic rs7517847 single nucleotide polymorphism (SNP) showed a significant frequency ($P=3.36x10^{-13}$) in the Jewish population (5) and in a Hungarian cohort (6). The uncommon coding mutation (rs11209026) on chromosome 1, a G to A transition (Arg381Gln), has been shown to confer a strong protection against CD in case-control and

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family-based studies of Caucasian and Jewish cohorts with ileal CD (7). The association of this variant with CD appears to be correlated also in Swedish patients with IBD and not in linkage disequilibrium (r^2=0.03) (8). The role attributed to *IL23R* is not to actively induce the differentiation of naive T cells, but rather to stabilise the already differentiated Th17 sub-population (9).

Evidence for genetic risk factors for CD is well established with respect to the *NOD2)/CARD15* gene (10). In 1996, Hugot *et al* discovered *NOD2/CARD15,* the first gene identified to be associated with IBD (11). Its discovery was one of the first success stories in complex polygenic disease genetics and has been responsible for a renewed interest in the innate immune response in IBD. *NOD2/CARD15* is a member of a family of intracellular pattern-recognition receptors (PRRs) that recognise the micro-bial component muramyl dipeptide (MDP), a degradation product of peptidoglycan derived from the cell wall of gram-negative and gram-positive bacteria (12). The association of the *NOD2/CARD15* variant with the disease phenotype has been primarily observed in ileal disease and in this respect is important in the reduced secretion of γ-defensins by ileal Paneth cells in patients carrying the *NOD2/CARD15* rs2066845 variant, which accounts for about 80% of *NOD2* variants associated with CD disease (13). Functional analyses of human peripheral mononuclear cells point to a loss of function due to CD-associated polymorphisms, related to an MDP-sensing defect, showing that *NOD2/CARD15* is a member of the innate immunity signalling pathways (14).

In a European genome-wide association study the coding variants rs2241879 and rs2241880 within the *ATG16L1* gene were reported to be highly associated with CD and to account for the whole disease risk exerted by this locus (15). The association of the *ATG16L1* gene with CD has been shown in numerous studies (16-18). *ATG16L1* is part of a family of genes implicated in autophagy, a biological process involved in protein degradation, antigen processing, regulation of cell signalling and many other pathways essential for the initiation and regulation of the inflammatory response. The association of the *ATG16L1* gene with CD suggests that autophagy is likely to have an important role in disease pathogenesis (19).

Recently, a North American genome-wide association study identified a novel gene variant of *PHOX2B* as well as one SNP (rs16853571) that were associated with CD. The DNA-associated protein encoded by this gene is a member of the paired family of homeobox proteins localized in the nucleus. This protein functions as a transcription factor involved in the development of several major noradrenergic neuron populations and in the determination of a neurotransmitter phenotype (20).

The aim of the present study was to investigate the association between CD susceptibility and variants of 4 candidate genes, namely *IL23R* (rs7517847 and rs11209026 variants), *NOD2/CARD15* (rs2066845), *ATG16L1* (rs2241879 and rs2241880 variants) and *PHOX2B* (rs16853571) in a sample of Italian patients. In addition, we examined the correlation of the genotype with specific disease sub-phenotypes, including gender, smoking habits, age, appendectomy, disease behaviour at diagnosis, disease severity, extra-intestinal manifestations and the possible genetic interaction. For this purpose, 19 Caucasian Italian patients affected by CD and undergoing regular follow-up at the Gastroenterology Unit of Policlinico Sant'Orsola, University of Bologna, were studied. The diagnosis of CD was carried out by conventional endoscopic, histological, and radiological criteria. Patients affected by other concomitant autoimmune diseases and patients/controls that did not complete the study protocol were excluded. The phenotype classification of CD was based on the disease localization and behaviour, according to the Montreal classification.

Materials and methods

Study population. The study population included 19 Italian patients with CD and 20 Italian healthy, unrelated controls. Patients were consecutively recruited from the unit of General Surgery-Taffurelli, Policlinico Sant'Orsola, University of Bologna, Italy. The study protocol was approved by the Ethics committee of Policlinico Sant'Orsola. Informed consent was obtained from each participant. Diagnosis of CD was established according to accepted clinical, endoscopic radiological and histological criteria (21). Patients were asked to complete a detailed clinical questionnaire concerning different features of the disease (Table I). The following data were collected from CD patients: gender, age at diagnosis, disease duration, family history of inflammatory bowel disease (IBD) (it was considered familial, if one first or second-degree relative was also affected), smoking habits, appendectomy, disease localization at diagnosis, current localization of the disease, clinical behaviour at diagnosis, changes in clinical behaviour, current clinical behaviour, severity of disease, surgery and extra-intestinal manifestations.

Disease localization was defined as the maximum extent of digestive tract involvement at the latest follow-up. Patients were recruited when IBD was confirmed, and they had undergone full colonoscopy with biopsy and/or surgical resection. A group of 20 healthy unrelated subjects (mainly students, blood donors and hospital employees) were selected as controls.

DNA extraction. Genomic DNA was isolated from 2 ml of peripheral blood anticoagulated with EDTA as previously described (22). DNA was extracted with the QIAamp DNA Blood kit (Qiagen), according to the manufacturer's protocols. The DNA quality was checked on a standard 1% agarose gel and quantified with the GelDoc 2000 system (Bio-Rad, Hercules, CA). DNA samples of the patients and control subjects were analysed for the following gene variants: *NOD2/CARD15* rs2066845, *ATG16L1* rs2241879 and rs2241880, *IL23R* rs7517847 and rs11209026 and *PHOX2B* rs16853571.

Genotyping of the mutations. Mutations were detected by polymerase chain reaction (PCR) followed by automatic sequencing. The primers for amplification were designed using the software Amplify, following standard criteria (23). The sequences for the specific PCR primers for each SNP are given in Table II. PCR experiments were performed in a final volume of 25 μl, containing 2 μg of sample, 1 unit Taq Polymerase (Takara, Shiga, Japan) with companion reagents (0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer), and 0.4 mM of each primer. An initial denaturation step of 2 min at 94˚C was followed

Table I. Clinical data of the Italian CD patients investigated.

Patient	Gender	Age at diagnosis[a]	Disease duration[b]	Family history of IBD[c]	Smoker[d]	Appendectomy surgery	Disease localization at diagnosis[e]	Current localization of disease[e]	Clinical behaviour at diagnosis[f]	Changes in clinical behaviour[g]	Current clinical behaviour[h]	Severity of disease[i]	Surgical[j]	Extra-intestinal manifestations
50014	F	A2	25	No	Ex	Yes	L3	L1	B1	B1-B2-B3	B1	6	Yes	Yes
50015	F	A2	24	No	No	No	L2	L2	B1	B1-P	B3-P	6	Yes	Yes
50024	M	A2	22	No	Ex	No	L2	L3	B1	B1-P-B2	B1	6	Yes	No/Yes
50034	M	A1	28	No	No	No	L3	L2	P+B1	Cancer sub-occlusion	B3-P	5	Yes	No
50052	M	A3	9	No	Ex	Yes	L1	L3	B2	After surgery	B1	5	Yes	Yes
50056	M	A2	32	No	Ex	Yes	L1	L1	B2	B2-B3	B1	6	Yes	No
50064	M	A3	5	No	Yes	No	L1	L1	B1	B1	B1	4	No	Yes
50070	F	A2	4	No	No	Yes	L3	L3	B1	B1-B2-B3	B1	6	Yes	No
50082	F	A1	22	No	Yes	Yes	L2	L3	B1	B1-B2-B3-P	B1	6	Yes	Yes
50084	F	A2	14	No	Ex	No	L3	L2	B1	B1-B3-P	B1-P	6	Yes	Yes
50090	F	A2	7	No	Yes	No	L1	L1	B2	B2-B3	B1	6	Yes	No
50120	M	A3	4	No	No	No	L1	L1	B2	B2	B2	6	Yes	No
50144	F	A2	5	No	Ex	Yes	L3	0 (L1)	B1	B1-B2-B3	B1	5	Yes	Yes
50170	M	A1	7		No	No	L1/L3	L1	B1	B1-B2-B3	B1	6	Yes	No
50185	M	A2	1	No	Ex	No	L3	L3	B2	B2-B3	B1	6	Yes	Yes
50233	M	A2	24	No	No	No	L1	L1	B2	B2-B3	B1	6	Yes	No
50234	M	A3	6	Yes	Ex	Yes	L1	L1	B2	B1	B1	6	Yes	No
50243	F	A3	0.5	No	Ex	Yes	L1	L1	B2	B1	B1	6	Yes	No
50253	F	A2	1	No	Ex	No	L1	L1	B2	B1	B1	6	Yes	No

M, male; F, female. [a] A1, <16; A2, 17-40; A3, >40 years. [b]Time lag between diagnosis and the present time, expressed in years. [c]1st degree relatives. [d]Ex, ex-smoker; Yes, current smoker; No, never smoked. [e]L1, ileal; L2, colic; L3, ileocolic; L4, upper gastrointestinal. [f-h]B1, not fistulising, no stenosis; B2, stenosis; B3, fistulising; P, perianal disease. [i]Following the classification of Silverstein *et al*: 4, severe refractory therapy with immunotherapy and biological drugs but no surgery; 5, surgery without immunotherpay or biological drugs; 6, surgery and also immunotherapy and biological drugs (32). [j]Surgical therapy from diagnosis at the present time.

Table II. Primers used for PCR amplification.

Primer	Sequences 5'→3'
IL23R rs7517847 L	ATTGACATTCCCTTCATACCTACCA
IL23R rs7517847 R	AGGAGACAGCCCATAAAGATACAAA
IL23R rs11209026 L	AATGATCGTCTTTGCTGTTATGTTG
IL23R rs11209026 R	CATTGTTCTTTTTATTTTCCTTTATCT
NOD2 rs2066845 L	TCTGGCTGGGACTGCAGAGG
NOD2 rs2066845 R	CTGAAGCCTTGGGTGATCAC
ATG16L1 rs2241879 L	AGAGCCAAAAGGTGGAAAGG
ATG16L1 rs2241879 R	GGATACTCATCCTGGTTCTGGTAAA
ATG16L1 rs224188 L	ATTTGTCTTTATGTTATTTCTTAGGAGACG
ATG16L1 rs2241880 R	GGTCCTTTCTAGGCCTGCA
PHOX2B rs16853571 L	ATCGTCCAAGTTTCTTCGTTCC
PHOX2B rs16853571 R	GAAATTCATCACCTCCTGACCAC
B2M L	GTGGGATCGAGACATGTAAGCAGC
B2M R	CTCCTAGAGCTACCTGTGGAGCAA

Source sequences for primer design were: NM_144701.2 (*IL23R*), No. NM_022162.1 (*NOD2*), No. NM_030803.6 (*ATG16L1*). No. NM_003924.3 (*PHOX2B*) and No. AB021288 (*B2M*).

by amplification for 40 cycles (30 sec at 94˚C, 30 sec at 61˚C, 30 sec at 72˚C) and a final extension for 7 min at 72˚C. All the PCR products obtained were analysed by gel electrophoresis following standard methods: ethidium bromide incorporation in the gel (0.5 mg/ml final concentration), and photography by the GelDoc 2000 system (Bio-Rad).

Wild-type/mutant genotype was confirmed by automatic sequencing using the ABI-PRISM 310 BigDye™ Terminator v.3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA, USA). The sequencing products were purified using DyeEx Spin kits (Qiagen) and visualized on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems). About 100-150 bp of the target gene were amplified and about 10 bp around the SNP were also analysed in order to identify new possible SNPs. This method allowed us to be aware of any new mutation around the studied SNP. Two different operators read the sense and antisense sequences, and a random check was performed by a third operator.

Statistical analysis. Allele and genotype frequencies in patients and in controls were compared by the Fisher's exact test; P-values were considered significant at a level ≤0.05. The odds ratios (OR) and P-values were calculated using a website tool (http://faculty.vassar.edu/lowry/VassarStats.html). The 95% confidence intervals were calculated for the OR.

Allele frequencies of controls were tested for the Hardy-Weinberg equilibrium, by comparing the expected and observed genotypes by the Fisher's exact test. All markers showed no statistically significant deviation from the Hardy-Weinberg equilibrium in both normal subjects and CD patients, except for the *IL23R* rs7517847 variant in CD patients (P=0.017).

Results

Genotyping was assessed by automatic sequencing of the *IL23R*, *NOD2/CARD15*, *PHOX2B* and *ATG16L1* genes. About 10 bp around the SNP were also analysed, but no new mutations were identified.

Genotyping of the rs7517847 and rs11209026 variants within IL23R in CD patients. Twenty healthy subjects were genotyped for *IL23R* rs7517847 and rs11209026 along with 18 and 19 CD patients, respectively (Table III). The rs7517847 risk allele G frequency was increased in CD subjects (42%) compared with controls (20%) (OR, 2.9; 95% CI, 1.06-7.9; P=0.03). Furthermore, *IL23R* rs7517847 was found to be associated with CD in the homozygous (GG) condition (OR, 8.7; 95% CI, 0.9-81.6; P=0.038).

A total of 18 patients were genotyped for *IL23R* rs11209026 polymorphism. The protective allele (A) the frequency was 13% in normal subjects vs. 3% in CD patients, but we did not find a significant association. The homozygous form AA was absent either in normal or CD subjects. When we stratified for the carrier risk allele G rs7517847 we found a strong association with the protective allele A rs11209026 in normal subjects (57% compared to 10% in CD patients; OR, 0.08; 95% CI, 0.0065-1.0687; P=0.05).

Genotyping of NOD2/CARD15 rs2066845 and PHOX2B rs16853571. The evaluation of *NOD2/CARD15* (rs2066845) and *PHOX2B* (rs16853571) polymorphisms was available in 20 healthy control and in 19 and 16 CD patients, respectively (Table III). Our study did not show a significant association of the risk alleles C of either gene, with CD disease. No carriers

Table III. Allele and genotype frequency analysis of the six investigated gene variants.

	Control	CD	P-value*	Odds ratio (95% CI)
ATG16L1 rs2241880				
Wild-type (AA)	3 (15%)	3 (17%)	0.6	1.10 (0.19-6.4)
Heterozygous (AG)	11 (55%)	9 (50%)	0.5	0.80 (0.2-2.9)
Homozygous (GG)	6 (30%)	6 (33%)	0.5	1.10 (0.2-4.5)
n	20	18		
Allele G (risk) frequency	57%	58%	0.56	1.03 (0.4-2.5)
ATG16L1 rs2241879				
Wild-type (TT)	6 (30%)	7 (37%)	0.45	1.36 (0.35-5.1)
Heterozygous (CT)	11 (55%)	9 (47%)	0.43	0.70 (0.2-2.5)
Homozygous (CC)	3 (15%)	3 (16%)	0.64	1.06 (0.18-6.05)
n	20	19		
Allele C (risk) frequency	43%	39%	0.48	0.88 (0.35-2.17)
PHOX2B rs16853571				
Wild-type (AA)	17 (85%)	15 (94%)	0.39	2.60 (0.2-28.2)
Heterozygous (AC)	3 (15%)	1 (6%)	0.39	0.30 (0.03-4.03)
Homozygous (CC)	0 (0%)	0 (0%)	1	–
n	20	16		
Allele C (risk) frequency	7%	3%	0.39	0.30 (0.03-4.02)
NOD2 rs2066845				
Wild-type (GG)	19 (95%)	18 (95%)	0.7	0.94 (0.05-16.3)
Heterozygous(GC)	1 (95%)	1 (5%)	0.7	1.05 (0.06-18.1)
Homozygous (CC)	0 (0%)	0 (0%)	1	–
n	20	19		
Allele C (risk) frequency	3%	3%	0.7	1.05 (0.06-17.4)
IL23R rs7517847				
Wild-type (TT)	13 (65%)	9 (47%)	0.2	0.48 (0.13-1.75)
Heterozygous(TG)	6 (30%)	4 (21%)	0.39	0.60 (0.14-2.6)
Homozygous (GG)	1 (5%)	6 (32%)	0.038	8.70 (0.9-81.6)
n	20	19		
Allele G (risk) frequency	20%	42%	0.03	2.90 (1.06-7.9)
IL23R rs11209026				
Wild-type (GG)	15 (75%)	17 (94%)	0.11	5.60 (0.59-54)
Heterozygous(GA)	5 (25%)	1 (6%)	0.11	0.17 (0.01-1.6)
Homozygous (AA)	0 (0%)	0 (0%)	1	–
n	20	18		
Allele A (protective) frequency	13%	3%	0.12	0.20 (0.02-1.8)

*P-value according to the Fisher's exact test.

of the risk homozygous condition CC were found in the normal subjects or in the CD patients of either SNPs.

Genotyping of the ATG16L1 rs2241879 and rs2241880 variants in CD patients. The evaluation of *ATG16L1* polymorphisms rs2241879 and rs2241880 was available in 20 healthy subjects and in 19 and 18 CD patients, respectively (Table III). Our results did not show a statistically significant association of the risk alleles C rs2241879 (39% frequency in CD patients vs. 43% in the control group) or G rs2241880 (58% frequency in CD vs. 57% in the control group) with CD disease. Accordingly, by comparing genotype frequencies no significant increase in carriers of the rs2241880 homozygous GG risk condition was found in CD patients in comparison to normal subjects (OR, 1.03; 95% CI, 0.4-2.5; P=0.56). Similarly, the rs2241879 homozygous CC genotype was not significantly correlated to CD.

Genotype-phenotype correlation. The correlation of the *ATG16L1* rs2241879 and rs2241880 SNPs and of the *IL23R*

Table IV. Correlation of the *ATG16L1* rs2241879 SNP frequency with sub-phenotypes within CD patients.

ATG16L1	SNP rs2241879	Wild-type	P-value[b]	OR; 95% CI
Gender			P=0.40	
M	7	3		
F	5	4		
Smoker			P=0.03	–
Yes[a]	6	7		
No	6	0		
Appendectomy			P=0.20	
Yes	4	4		
No	8	3		
Age			P=0.70	
A1	2	1		
A2+A3	10	6		
Behaviour at diagnosis			P=0.57	
B1	6	4		
B2	6	3		
Disease severity			P=0.10	
4-5	1	3		
6	11	4		
Extra-intestinal manifestations			P=0.006	0.03 (0.002-0.45)
Yes	2	6		
No	10	1		

[a]This group includes smokers and ex-smokers; [b]Fisher's exact test.

Table V. Correlation of the *ATG16L1* rs2241880 SNP frequency with sub-phenotypes within CD patients.

ATG16L1	SNP rs2241880	Wild-type	P-value[b]
Gender			P=0.10
M	6	3	
F	9	0	
Smoker			P=0.50
Yes[a]	11	1	
No	4	1	
Appendectomy			P=0.20
Yes	7	0	
No	8	3	
Age			P=0.44
A1	2	1	
A2+A3	13	2	
Behaviour at diagnosis			P=0.60
B1	6	2	
B2	7	3	
Disease severity			P=0.40
4-5	2	1	
6	13	2	
Extra-intestinal manifestations			P=0.20
Yes	7	0	
No	8	3	

[a]This group includes smokers and ex-smokers; [b]Fisher's exact test.

Table VI. Correlation of the *IL23R* rs7517847 SNP with sub-phenotypes within CD patients.

IL23R	SNP rs7517847	Wild-type	P-value[b]
Gender			P=0.58
M	5	4	
F	5	4	
Smoker			P=0.50
Yes[a]	8	5	
No	1	4	
Appendectomy			P=0.39
Yes	5	3	
No	5	6	
Age			P=0.54
A1	2	1	
A2+A3	8	8	
Behaviour at diagnosis			P=0.20
B1	4	6	
B2	6	3	
Disease severity			P=0.60
4-5	3	2	
6	6	8	
Extra-intestinal manifestations			P=0.47
Yes	5	3	
No	5	5	

[a]This group includes smokers and ex-smokers; [b]Fisher's exact test.

rs7517847 SNP to several phenotypic manifestations, namely gender, age, appendectomy, behaviour at diagnosis, disease severity and extra-intestinal manifestations were investigated in CD patients (Table IV-VI). For the purpose of this study, we grouped together smokers and ex-smokers.

The stratification of patients in a smoking/non-smoking phenotype, and the association with the *ATG16L1* rs2241879 variant revealed an association with smoking (P=0.03; Table IV), so that smoking seems to exert an additional effect to SNP in the development of CD. Moreover, we found an association in our patients of the G risk allele with the lack of extra-intestinal manifestations (OR, 0.03; 95% CI 0.002-0.45; P=0.006). This allele shows a protective correlation with respect to the CD phenotype of extra-intestinal manifestations.

Discussion

Recently, with the massive use of genome-wide association technology, several novel genes and loci involved in the pathogenesis of IBD and specifically of CD have been identified. In the present study, we investigated the prevalence of *IL23R, NOD2/CARD15, ATG16L1* and *PHOX2B* genetic variants in a group of Italian CD patients. Moreover, we compared the results with clinical phenotype characteristics in order to identify a possible genotype-phenotype association.

We did not find any association of CD with *NOD2/CARD15, PHOX2B* and *ATG16L1*. This is in contrast with previously reported associations of *NOD2* and *ATG16L1* alleles with CD. A possible explanation could be the size of the investigated sample, so that further analysis in a larger number of Italian patients will be necessary to confirm a lack of association. However, our data are in agreement with the literature about the controversial role of the *NOD2/CARD15* and *ATG16L1* SNPs in the pathogenesis of CD. The *NOD2/CARD15* mutation is absent in Asian CD patients as well as in the normal healthy population (24-26). These findings indicate that the *NOD2/CARD15* is not the major contributor to CD susceptibility in the Japanese population. Similar data have been found in Turkish patients with IBD (27). Again, a lack of association between *ATG16L1* and CD was previously observed in an Italian CD population study, in which Perricone *et al* failed to find a significant association between the candidate genetic variation *ATG16L1* rs2241880 and CD (28). Therefore, our results could reinforce the notion of a different relevance of *NOD2/CARD15* and *ATG16L1* in the pathogenesis of CD in patients of different geographical origin, with a limited role in the Italian population.

While no differences were noted between disease and normal conditions in the *ATG16L1* rs2241879 allele frequency, in our study this polymorphism revealed a significant association with smoking in patients with CD. Interestingly, in this case none of the non-smokers presented the wild type allele, possibly suggesting the prevalence of a genetic contribution in the absence of the smoking environmental factor.

In our study we grouped together smokers and ex-smokers, assuming that the contribution of smoking habits to the development or worsening of CD requires time to take effect. Smoking is a well-established risk factor for CD, and several

researchers have found a strong correlation between smoking and CD, particularly with the late-onset CD patients (age of diagnosis >40 years) (29).

Moreover, the results of the genotype-phenotype correlations demonstrate a strong correlation of the *ATG16L1* rs2241879 investigated SNP with another aspect of CD phenotype, that is with the lack of extra-intestinal manifestations (P=0.006). This finding suggests that the *ATG16L1* variant rs2241879 may exert a protective effect on the development of extra-intestinal manifestations in CD patients. *ATG16L1* is involved in autophagy, a process recently shown to be essential for immune tolerance (30). It may be hypothesized that, in a certain genomic and functional context, the *ATG16L1* rs2241879 SNP may protect from multi-organ inflammation by modulating the generation of a self-tolerant T-cell repertoire through autophagy.

Interestingly, despite the fact that the size of the investigated sample was not large, we were able to find a significant association of the *IL23R* rs7517847 SNP with CD genotype, in homozygous condition (P<0.05). However, we did not find an association of the same SNP with any disease sub-phenotype. This result confirms previous studies on North American (31) and Italian populations (28).

IL23R gene variants have now been repeatedly implicated in a number of inflammation diseases, suggesting that these pathologies might be mediated by the Th17 pathway. Our results provide an independent confirmation of the association between the candidate genetic variation *IL23R* and CD (10), and reinforce the role of this new polymorphism as a genetic determinant of CD. Further research is necessary to understand how *IL23R* contributes to disease susceptibility in CD. Nevertheless, our data do not show a correlation of *IL23R* with the phenotype, as previously reported (31).

This is the first study showing an association of *ATG16L1* variants with smoking habits in patients with CD, and with the development of extra-intestinal manifestations as a protective factor. Further studies are needed to verify if these findings may be extended to different patient populations. Our report confirms variability in genetic factors associated with CD across different populations and in different patients, as it is expected for a multifactorial disease with a complex interplay of environmental and genetic components.

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