



## Replication of *interleukin 23 receptor* and *autophagy-related 16-like 1* association in adult- and pediatric-onset inflammatory bowel disease in Italy

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19 years), 749 healthy controls, and 546 healthy parents (273 trios) were included in the study. The rs2241880 [*autophagy-related 16-like 1 (ATG16L1)*], rs11209026 and rs7517847 [*interleukin 23 receptor (IL23R)*], rs2066844, rs2066845, rs2066847 (*CARD15*), rs1050152 (*OCTN1*), and rs2631367 (*OCTN2*) gene variants were genotyped.

**RESULTS:** The frequency of G allele of *ATG16L1* SNP (Ala197Thr) was increased in patients with CD compared with controls (59% vs 54% respectively) (OR = 1.25, CI = 1.08-1.45,  $P = 0.003$ ), but not in UC (55%). The frequency of A and G (minor) alleles of Arg381Gln, rs11209026 and rs7517847 variants of *IL23R* were reduced significantly in CD (4%, OR = 0.62, CI = 0.45-0.87,  $P = 0.005$ ; 28%, OR = 0.64, CI = 0.55-0.75,  $P < 0.01$ ), compared with controls (6% and 38%, respectively). The A allele (but not G) was also reduced significantly in UC (4%, OR = 0.69, CI = 0.5-0.94,  $P = 0.019$ ). No association was demonstrated with sub-phenotypes and interaction with *CARD15*, and *OCTN1/2* genes, although both gene variants were associated with pediatric-onset disease.

**CONCLUSION:** The present study confirms the association of *IL23R* polymorphisms with IBD, and *ATG16L1* with CD, in both adult- and pediatric-onset subsets in our study population.

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**Key words:** Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Genetic predisposition; *Autophagy-related 16-like 1*; *Interleukin 23 receptor*; Genome-wide association study; Pediatric inflammatory bowel disease

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### Abstract

**AIM:** To investigate gene variants in a large Italian inflammatory bowel disease (IBD) cohort, and to analyze the correlation of sub-phenotypes (including age at diagnosis) and epistatic interaction with other IBD genes.

**METHODS:** Total of 763 patients with Crohn's disease (CD, 189 diagnosed at age < 19 years), 843 with ulcerative colitis (UC, 179 diagnosed <

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a polygenic trait that includes two similar, yet distinct conditions, namely Crohn's disease (CD) and ulcerative colitis (UC)<sup>[1]</sup>. It is widely accepted that both diseases result from an inappropriate response of a defective mucosal immune system to indigenous flora and other luminal agents in a genetically susceptible host<sup>[2]</sup>.

Eleven IBD genome-wide linkage analyses in families with multiple IBD affected members, as well as two different meta-analyses<sup>[3,4]</sup> have identified several linkage regions<sup>[5]</sup>. Following the identification of *NOD2* (or *CARD15*), the first gene contributing to CD susceptibility (IBD1 locus), further fine mapping studies have identified a risk haplotype (IBD5 locus) on chromosome 5q<sup>[6]</sup>, along with two polymorphisms in the solute carrier family 22A4/22A5 (*SLC22A4/A5*) coding for *OCTN1* and *OCTN2*, suggested as candidate genes<sup>[7]</sup>, and another polymorphism on the *disk large homolog 5* (*DLG5*) gene<sup>[8]</sup>. However, these risk-associated variants and several others reported<sup>[9-13]</sup> with conflicting results, explain only a minor component of the genetic risk in IBD.

Whole genome association (GWA) studies in IBD have rapidly led to the identification of novel susceptibility loci associated with CD<sup>[14]</sup>, such as *interleukin-23 receptor* (*IL23R*) and *autophagy-related 16-like 1* gene (*ATG16L1*). An uncommon coding mutation (rs11209026) in the *IL23R* gene<sup>[15]</sup> on chromosome 1 (1p32.1-p31-2), a G-to-A transition at nucleotide 1142 (Arg381Gln), has been identified to confer strong protection against CD in case-control ( $P = 5.05 \times 10^{-9}$ ) and family-based studies of Caucasian and Jewish cohorts with ileal CD. Further, GWA<sup>[16-19]</sup> and replication studies<sup>[1,2]</sup> have consistently confirmed strong association between variations at *IL23R* and CD. The intronic rs7517847 SNP (single nucleotide polymorphism) gave the most significant signal ( $P = 3.36 \times 10^{-13}$ )<sup>[15]</sup>. The association of this variant appeared statistically independent from Arg381Gln and not in linkage disequilibrium ( $r^2 = 0.03$ )<sup>[15]</sup>. A large number of replications in adult-<sup>[20-24]</sup> and pediatric-onset<sup>[25-29]</sup> cohorts have been reported, but no significant association with specific CD sub-phenotype has been identified. Moreover, an association with UC has also been observed<sup>[23,24,28,30,31]</sup>.

In a recent German GWA scan<sup>[32]</sup>, the non-synonymous rs2241880 (Ala197Thr) variant of *ATG16L1* on chromosome 2p37.1 was found to be associated with CD, and appeared to account for all of the disease risk conferred by this locus. This association has been consistently demonstrated in a number of independent studies in adult<sup>[24,33-36]</sup> but with conflicting data in pediatric<sup>[34,37,38]</sup> IBD cohorts.

The aims of the present study were to investigate the association between variants of two candidate genes

*IL23R* and *ATG16L1* and IBD in an Italian cohort. In addition, we examined the genotype-phenotype correlation with specific disease subtypes, including the early onset of disease, and the possible genetic interactions with *OCTN1/2*, and *CARD15* genes.

## MATERIALS AND METHODS

### Materials

The study population included 763 CD and 843 UC unrelated patients recruited from four referral centers for adult IBD: the "Casa Sollievo della Sofferenza" Hospital of San Giovanni Rotondo, and the University Hospitals of Padua, Naples and Milan. In addition, 368 patients with pediatric onset of disease (age at diagnosis < 19 years) were included in the study because of a multi-center effort of the Italian Society of Pediatric Gastroenterology, Hepatology and Nutrition (SIGENP). This study cohort has been reported elsewhere<sup>[39]</sup>. Two hundred and seventy-three IBD patients also had both parents available for the purpose of a family-based analysis. The 749 healthy controls (415 male, mean age  $43 \pm 11$  years, range 22-75) were randomly recruited from three sites: San Giovanni Rotondo (Southern Italy,  $n = 451$ ), Rome (Central Italy,  $n = 114$ ) and Milano (Northern Italy,  $n = 184$ ), in order to minimize potential geographic heterogeneity. These subjects comprised of unrelated, asymptomatic individuals (blood donors, students, and staff members), all Caucasians with no Jewish descent. The study protocol was approved by the local Ethics Committees, and a written informed consent was obtained from each subject (or parents).

Demographic and sub-phenotype data of IBD patients are presented on Table 1. The diagnosis of CD and UC was established according to accepted clinical, endoscopic, radiological, and histological criteria<sup>[1]</sup>. The Montreal<sup>[40]</sup> classification was used for CD, based on the age at diagnosis (A), location (L), and disease behavior (B). In patients with UC, the disease location was categorized according to the Montreal classification, by distinguishing ulcerative proctitis (E1), left-side colitis (E2), and extensive colitis (E3). Patients with indeterminate colitis were excluded from the study. In all patients, the following clinical features were recorded: family history, age at diagnosis, duration of follow-up, presence of perianal fistulae, extraintestinal manifestations (presence or absence of any extraintestinal manifestation), previous abdominal surgery (either colectomy in UC or bowel resection in CD), and smoking habit (at least 1 cigarette/d). To account for the known modification of clinical characteristics during the disease course, only patients with at least two years of follow-up from the time of a confirmed diagnosis were included in the genotype/phenotype analysis.

### Methods

Genotyping of rs11209026 (*IL23R*), rs7517847 (*IL23R*), rs2241880 (*ATG16L1*), rs10150152 (*OCTN1*) and rs2631367 (*OCTN2*)<sup>[41]</sup> SNPs was performed using Applied Biosystems 7700 TaqMan assay

**Table 1** Demographic and clinical features of CD and UC according to the Montreal classification<sup>[39]</sup>

	CD (n = 763)	UC (n = 843)
Sex (M/F)	435/328	492/351
Duration of follow-up, mean ± SD (range)	8 ± 7 (1-37)	9 ± 7 (1-41)
Age at diagnosis (yr), mean ± SD (range)	29 ± 15 (1-79)	32 ± 16 (1-76)
≤ 16 (A1)	173 (22%)	155 (18%)
17-40 (A2)	431 (57%)	458 (55%)
> 40 (A3)	159 (21%)	230 (27%)
Disease localization CD, n (%)		
Ileum (L1 ± L4)	241 (31%)	
Colon (L2 ± L4)	200 (26%)	
Ileo-colon (L3 ± L4)	315 (41%)	
Upper GI (L4)	7 (2%)	
Disease extent UC, n (%)		
Rectum (E1)		102 (13%)
Left colon (E2)		416 (49%)
Pancolitis (E3)		325 (38%)
Disease behavior CD, n (%)		
Inflammatory (B1 ± p)	346 (46%)	
Stricturing (B2 ± p)	177 (23%)	
Penetrating (B3 ± p)	240 (31%)	
Perianal disease y/n (%)	137/626 (18%)	17/826 (2%)
Smoking history		
Yes	242 (33%)	119 (15%)
No	423 (55%)	510 (60%)
Ex	98 (12%)	214 (25%)
Family history of IBD y/n (%)	74/689 (10%)	53/790 (6%)
Surgery y/n, n (%)	237/526 (31%)	95/748 (13%)
Extra-intestinal manifestations y/n (%)	304/459 (40%)	182/661 (21%)

(Applied Biosystems, Foster City, CA). The *CARD15* variants were detected by means of Denaturing High Performance Liquid Chromatography (DHPLC) (Wave System, Transgenomic Ltd, UK) (R702W, L1007fsinsC) and RFLP (G908R), respectively, as described previously<sup>[39]</sup>. PCR reactions were carried out in 96-well plates on ABI 9700 Thermocyclers (Applied Biosystems, Foster City, CA). All samples were genotyped at the Molecular Laboratory of Gastroenterology Unit in San Giovanni Rotondo Hospital, Italy.

For the purpose of statistical analysis, after genotyping the markers, the Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotypes in  $2 \times 3$   $\chi^2$  tables. All markers showed no deviation from the Hardy-Weinberg equilibrium in controls ( $P > 0.05$ ). Allele-genotype frequencies and genotype/phenotype association were analysed by  $\chi^2$  and Fisher exact tests, when appropriate, by the SPSS software ver 1.5. The Hardy-Weinberg equilibrium and marker linkage disequilibrium analysis were also performed by the Arlequin software ver 2.0. Pairwise SNP linkage disequilibrium (LD) coefficients, haplotype frequencies, and Transmission Disequilibrium Test (TDT) were estimated using Haploview<sup>[42]</sup>. Logistic regression analysis was used to assess the conditional independence between genotypes and IBD phenotypes and to test for gene-gene interactions. The frequencies and odds ratios of individual *CARD15* genotypes were stratified by *ATG16L1* (rs2241880) and *IL23R* (rs1209026); and *IL23R* genotypes were stratified by *ATG16L1* genotypes. An interaction was considered significant at  $P < 0.05$ .

## RESULTS

### Patients and controls

A total of 2901 subjects were investigated. These comprised of 763 patients with CD (435 males and 328 females), with a mean age at diagnosis of 29 years (range 1-79), and 843 patients with UC (492 males, 351 female), with a mean age at diagnosis of 32 years (range 1-76 years). In addition, 749 healthy controls and 546 healthy parents were also evaluated. Of note, 368 patients (180 male) were diagnosed at the age of 18 years or less.

### Genotyping of *ATG16L1* variant in IBD patients

The evaluation of the T300A polymorphism (rs2241880) was available in 667 CD and 668 UC patients: the frequency of the G allele was increased in CD patients compared with controls (59% vs 54%) ( $P = 0.003$ , OR = 1.25, CI = 1.08-1.45) (Table 2). Accordingly, by comparing genotype frequencies (Table 2), a significant increase in carriers of G risk allele was found in CD patients compared to controls (84% vs 79%) ( $P = 0.008$ , OR = 1.44, CI = 1.10-1.89). After stratifying the CD cohorts on the basis of age at diagnosis (adult  $\geq 19$  years; pediatric  $< 19$  years), the allele/genotype difference remained significant only in the adult subgroup ( $P = 0.004$ ), perhaps due to the small sample size of the pediatric subgroup, although the frequencies were similar.

There was no significant difference in the allele and genotype frequencies between UC patients and controls for the groups as a whole and after stratifying on the basis of pediatric and adult age at diagnosis.

### Genotyping of *IL23R* variants in IBD patients

A total of 723 CD, 804 UC, and 716 controls were genotyped for the rs7517847 polymorphism. The minor allele (G) frequency was significantly reduced (28%) in CD cases compared with controls (38%) ( $P < 0.01$ , OR = 0.64, CI = 0.55-0.75), with a significant reduction in carriers (49% vs 61% in controls) ( $P < 0.01$ , OR = 0.63, CI = 0.51-0.77) (Table 2). These differences remained significant after stratification of the patients on the basis of age at diagnosis. In contrast, no significant difference in allele and genotype frequencies was found in UC patients (Table 2).

A total of 735 CD and 823 UC patients, and 726 healthy controls were genotyped for rs11209026 polymorphism. The minor allele frequency (A) in CD patients was 4%, compared with 6% in controls, yielding a protective OR of 0.62 (CI = 0.45-0.87;  $P = 0.005$ ) (Table 2). Compared with controls (87%), the frequency of the risk genotype was significantly increased in CD patients (92%) ( $P = 0.005$ , OR = 0.61, CI = 0.43-0.86). These differences resulted in similar statistical significance after stratifying CD patients on the basis of age at diagnosis.

In addition, a high significant association was also found in UC patients, with a similar MAF frequency (4%) leading to a protective OR of 0.69 (CI = 0.50-0.94;  $P = 0.019$ ). Accordingly, the frequency of GG genotype was also significantly increased in UC patients (91%,  $P = 0.015$ ).

**Table 2** Genotypes and alleles distribution for *ATG16L1* and *IL23R* SNPs in CD, UC and controls

	Genotypes					Alleles				
	AA	Aa	aa	Total	P	OR (95% CI)	Freq	P	OR (95% CI)	
<i>ATG16L1</i>										
<i>rs2241880</i>	(AA Aa vs aa)					G				
CD Total	227 (34%)	335 (50%)	105 (16%)	667	0.008	1.44 (1.10-1.89)	0.59	0.003	1.25 (1.08-1.45)	
CD adult	165 (34%)	254 (52%)	72 (15%)	491	0.004	1.57 (1.16-2.13)	0.59	0.004	1.27 (1.08-1.49)	
CD pediatric	62 (35%)	81 (46%)	33 (19%)	176	0.466	1.17 (0.77-1.77)	0.58	0.122	1.20 (0.95-1.52)	
UC Total	212 (32%)	315 (47%)	141 (21%)	668	0.956	1.01 (0.78-1.30)	0.55	0.381	1.07 (0.92-1.24)	
UC adult	157 (31%)	244 (48%)	105 (21%)	506	0.839	1.03 (0.78-1.36)	0.55	0.469	1.06 (0.90-1.25)	
UC pediatric	55 (34%)	71 (44%)	36 (22%)	162	0.779	0.94 (0.63-1.42)	0.56	0.473	1.09 (0.86-1.39)	
Controls	214 (29%)	376 (50%)	159 (21%)	749	-	-	0.54	-	-	
<i>IL23R</i>										
<i>rs7517847</i>	(Aa aa vs AA)					G				
CD Total	366 (51%)	305 (42%)	52 (7%)	723	< 0.01	0.63 (0.51-0.77)	0.28	< 0.01	0.64 (0.55-0.75)	
CD adult	273 (51%)	225 (42%)	42 (8%)	540	< 0.01	0.63 (0.50-0.79)	0.29	< 0.01	0.65 (0.55-0.78)	
CD pediatric	93 (51%)	80 (44%)	10 (5%)	183	< 0.01	0.62 (0.45-0.86)	0.27	< 0.01	0.61 (0.48-0.79)	
UC Total	326 (41%)	390 (49%)	88 (11%)	804	0.567	0.94 (0.77-1.16)	0.35	0.111	0.89 (0.76-1.03)	
UC adult	259 (41%)	301 (48%)	71 (11%)	631	0.468	0.92 (0.74-1.15)	0.35	0.121	0.88 (0.75-1.03)	
UC pediatric	67 (39%)	89 (51%)	17 (10%)	173	0.927	1.02 (0.72-1.43)	0.36	0.400	0.90 (0.70-1.15)	
Controls	280 (39%)	328 (46%)	108 (15%)	716	-	-	0.38	-	-	
<i>rs11209026</i>						A				
CD Total	675 (92%)	60 (8%)	0 (0%)	735	0.005	0.61 (0.43-0.86)	0.04	0.005	0.62 (0.45-0.87)	
CD adult	502 (91%)	50 (9%)	0 (0%)	552	0.042	0.69 (0.48-0.99)	0.05	0.041	0.69 (0.49-0.99)	
CD pediatric	173 (95%)	10 (5%)	0 (0%)	183	0.006	0.40 (0.20-0.78)	0.03	0.007	0.41 (0.21-0.80)	
UC Total	750 (91%)	72 (9%)	1 (0%)	823	0.015	0.67 (0.48 - 0.93)	0.04	0.019	0.69 (0.50-0.94)	
UC adult	589 (91%)	58 (9%)	1(0%)	648	0.035	0.69 (0.49-0.98)	0.05	0.043	0.71 (0.51-0.99)	
UC pediatric	161 (92%)	14 (8%)	0 (0%)	175	0.085	0.60 (0.33-1.08)	0.04	0.087	0.61 (0.34-1.08)	
Controls	634 (87%)	91 (13%)	1 (0%)	726	-	-	0.06	-	-	

"A" refers to wild type and "a" to mutated allele. Adult and pediatric subgroups are divided on the basis of the age at diagnosis ( $\geq 19$  years and  $< 19$  years, respectively).

**Table 3** Haplotypes frequency of he two *IL23R* variants in IBD patients and controls

Haplotype		IBD		CD		UC	
<i>rs7517847</i>	<i>rs11209026</i>	Cases/Controls (% Haplotype)	P	Cases/Controls (% Haplotype)	P	Cases/Controls (% Haplotype)	P
T	G	68/62	0.0032	71/62	< 0.01	65/62	0.2166
G	G	28/32	0.0548	25/32	< 0.01	31/32	0.7398
G	A	4/6	0.0121	4/6	0.0088	4/6	0.0578

(Table 2). These statistical differences remained after stratifying the cohort on the basis of age at diagnosis.

Haplotype association analysis of two *IL23R* variants, *rs7517847* and *rs11209026*, was also estimated (Table 3). An increase in TG haplotype frequency was observed in IBD (68%,  $P = 0.0032$ ) and CD patients (71%,  $P \leq 0.01$ ), compared with controls (62%).

**Family-based analysis**

Given the lack of a significant association of allele/genotype frequencies of the *ATG16L1* gene in the pediatric subset, and the availability of 273 pediatric IBD Trios (138 CD, and 135 UC), the T300A polymorphism was tested by transmission disequilibrium test; a significant over-transmission of the G allele was found in CD patients (T:U = 90:62;  $P = 0.023$ ), but not in UC (Table 4). With respect to *IL23R*, a significant over-transmission of the T allele of *rs7517847* (T:U = 137:101), and the G allele of *rs11209026* (T:U = 34:19) SNPs was observed in the whole IBD cohort ( $P = 0.0196$ ,  $P = 0.0394$ ; respectively), and more specifically in CD patients ( $P = 0.0015$ ,  $P = 0.0833$ , respectively). However,

the results in the UC subset did not reach statistical significance. An association between the *rs7517847*-*rs11209026* (TG) haplotype and IBD, CD, and UC was also tested using the TDT. Consistent evidence of over-transmission of this haplotype in the whole IBD cohorts (T:U = 141:100,  $P = 0.0084$ ) was maintained (Table 4).

**Genotype/phenotype correlation**

Analysis of the allele and genotype frequencies of the *rs2241880* SNP of the *ATG16L1* gene, showed no association with disease location, behaviour, and age at diagnosis based on the Montreal classification of CD cases (Table 5). More specifically, we observed the lowest frequency of the G allele in isolated colonic disease (55%), increasing to 60% in ileal and ileo-colonic disease, without a clear difference in adult and pediatric subsets (colonic disease: adult 55%, pediatric 54%). Similarly, there was no association with gender, smoking history, perianal fistulae, and presence of extra-intestinal manifestations. Moreover, no association was found with any specific sub-phenotypes of UC (Table 6).

Similarly, there was no correlation between risk



**Table 4** Transmission disequilibrium test (TDT) for rs7517847 and rs11209026 *IL23R* SNPs for IBD, CD, and UC

Marker	Over-trans	Haplotype	IBD (n = 273)			CD (n = 138)			UC (n = 135)		
			T	U	P	T	U	P	T	U	P
rs7517847	T		137	101	0.0196	74	40	0.0015	63	61	0.8575
rs11209026	G		34	19	0.0394	18	9	0.0833	16	10	0.2393
		TG	141	100	0.0084	52	60	0.0012	45	34	0.5947
		GG	97	123	0.0790	50	39	0.0098	28	28	0.9940
		GA	14	24	0.1088	12	20	0.0585	9	13	0.6555
		TA	5	10	0.1831	13	8	0.7037	-	-	-
rs2241880	G		-	-	-	90	62	0.0231	-	-	-

TDT haplotypes are also included. Data of over-transmitted allele are given, with counts of transmitted (T) and un-transmitted (U) alleles.

**Table 5** Genotype and allele frequency distribution of *ATG16L1* and *IL23R* SNPs in CD cases stratified by phenotypic subgroups, (n)

SNP/gene genotype	rs2241880 <i>ATG16L1</i>					rs7517847 <i>IL23R</i>					rs11209026 <i>IL23R</i>				
	AA (105)	AG (335)	GG (227)	Total (667)	Freq (G) 0.501	GG (52)	GT (305)	TT (366)	Total (723)	Freq (G) 0.283	AA (0)	AG (60)	GG (675)	Total (735)	Freq (A) 0.041
Sex															
Male	54	193	126	373	0.597	28	169	212	409	0.275	0	27	390	417	0.032
Female	51	142	101	294	0.585	24	136	154	314	0.293	0	33	285	318	0.052
Age at diagnosis (yr)															
≤ 16 (A1)	28	72	53	153	0.582	9	76	74	159	0.296	0	9	150	159	0.028
17-40 (A2)	56	180	123	359	0.593	24	160	209	393	0.265	0	29	378	407	0.036
> 40 (A3)	20	71	40	131	0.576	14	57	73	144	0.295	0	19	123	142	0.067
Disease localization															
Ileum (L1 ± L4)	28	97	67	192	0.602	12	86	118	216	0.255	0	21	200	221	0.048
Colon (L2 ± L4)	31	92	48	171	0.550	10	76	104	190	0.253	0	18	170	188	0.048
Ileo-colon (L3 ± L4)	43	131	101	275	0.605	25	131	129	285	0.318	0	19	276	295	0.032
Upper GI (L4)	2	4	1	7	0.429	0	2	4	6	0.167	0	0	6	6	0.000
Disease behavior															
Inflammatory (B1)	44	154	98	296	0.591	21	133	162	316	0.277	0	25	294	319	0.039
Stricturing (B2)	22	79	46	147	0.582	10	64	82	156	0.269	0	16	145	161	0.050
Penetrating (B3)	38	85	70	193	0.583	17	93	103	213	0.298	0	17	201	218	0.039
Perianal disease															
Yes	14	52	37	103	0.612	8	54	59	121	0.289	0	11	113	124	0.044
No	89	269	175	533	0.581	40	233	293	566	0.277	0	46	529	575	0.040
Smoking history															
No	57	168	116	341	0.587	24	149	191	364	0.271	0	29	339	368	0.039
Yes	32	105	71	208	0.594	20	100	107	227	0.308	0	20	213	233	0.043
Ex	14	39	30	83	0.596	3	41	49	93	0.253	0	8	88	96	0.042
Surgery															
Yes	34	91	68	193	0.588	15	83	113	211	0.268	0	16	202	218	0.037
No	70	231	149	450	0.588	32	211	241	484	0.284	0	42	447	489	0.043

Only patients with at least 2 years of follow-up from diagnosis were included.

alleles and genotypes of the *IL23R* genes with specific sub-phenotypes of CD (Table 5) and UC (Table 6). Of note, the frequency of A allele of rs11209026 was the lowest in patients diagnosed at < 16 years of age (2.8%), increasing to 3.6% in the age group of 17-40 years, and 6.7% in those over 40 years, but the differences did not reach statistical significance (Table 5). More specifically, despite the large number of pediatric onset IBD patients investigated, genotype/phenotype frequencies were similar after further stratifying IBD population into adult and pediatric age of onset of the disease (data not shown).

**Interaction between *ATG16L1*, *IL23R*, *CARD15*, and *OCTN1/2* genes**

Logistic regression analysis was used to evaluate the individual contributions of *CARD15* (at least 1 variant against wild type), *ATG16L1* (GG/AG vs AA), *IL23R*

rs11209026 (AG/AA vs GG) and *OCTN* (diplotype TTCC against the rest) and CD risk, and to test for statistical interaction. The analysis included 763 CD cases and 749 controls. Table 7 shows the results of the logistic regression model with the individual contributions of *CARD15*, *ATG16L1*, *IL23R*, and *OCTN* to disease risk, their contribution after adjustment for *CARD15* genotype, and the contribution of interactions with *CARD15*. The individual contribution of all the predisposing genes was confirmed. By contrast, there was no evidence of a statistical interaction between all loci ( $P = 0.428$ ), and *ATG16L1*, *IL23R*, and *OCTN* by pairs and triplets (data not showed).

**DISCUSSION**

With the recent introduction of the GWA technology,

**Table 6** Genotype and allele frequencies of *ATG16L1* and *IL23R* SNPs in UC cases stratified by phenotypic subgroups, (n)

SNP/gene genotype	rs2241880 <i>ATG16L1</i>					rs7517847 <i>IL23R</i>					rs11209026 <i>IL23R</i>				
	AA (141)	AG (315)	GG (212)	Total (668)	Freq (G) 0.553	GG (88)	GT (390)	TT (326)	Total (804)	Freq (G) 0.352	AA (1)	AG (72)	GG (750)	Total (823)	Freq (A) 0.045
Sex															
Male	77	183	112	372	0.547	41	236	185	462	0.344	0	45	432	477	0.047
Female	64	132	100	296	0.561	47	154	141	342	0.363	1	27	318	346	0.042
Age at diagnosis (yr)															
≤ 16 (A1)	30	58	48	136	0.566	15	76	49	140	0.379	0	11	129	140	0.039
17-40 (A2)	79	159	102	340	0.534	40	213	170	423	0.346	0	33	401	434	0.038
> 40 (A3)	24	84	55	163	0.595	28	86	89	203	0.350	1	25	184	210	0.064
Disease localization															
Rectum	18	41	15	74	0.480	13	38	39	90	0.356	1	8	80	89	0.056
Rectum-sigmoid	20	55	39	114	0.583	20	82	46	148	0.412	0	21	134	155	0.068
Left colon	41	101	64	206	0.556	27	104	100	231	0.342	0	19	215	234	0.041
Colon	54	104	86	244	0.566	24	148	124	296	0.331	0	22	283	305	0.036
Perianal disease															
Yes	3	3	6	12	0.625	0	6	8	14	0.214	0	2	13	15	0.067
No	129	298	198	625	0.555	82	367	301	750	0.354	1	67	699	767	0.045
Smoking history															
No	81	173	125	379	0.558	51	203	185	439	0.347	1	38	408	447	0.045
Yes	25	54	25	104	0.500	10	65	41	116	0.366	0	12	104	116	0.052
Ex	25	72	51	148	0.588	20	105	77	202	0.359	0	18	191	209	0.043
Surgery															
Yes	11	33	26	70	0.607	9	37	35	81	0.340	0	9	74	83	0.054
No	122	268	178	568	0.549	74	337	274	685	0.354	1	61	638	700	0.045

Only patients with at least 2 years of follow-up from diagnosis were included.

**Table 7** Logistic regression analysis

Genes	P	OR	95% CI
<i>CARD15</i>	2.34E-14	2.908	2.211-3.825
ATG	0.008	1.442	1.099-1.894
ATG (adj <i>CARD15</i> )	0.014	1.439	1.077-1.924
ATG* <i>CARD15</i>	0.692	0.851	0.382-1.894
<i>IL23</i>	0.005	0.613	0.435-0.863
<i>IL23</i> (adj <i>CARD15</i> )	0.029	0.629	0.414-0.954
<i>IL23</i> * <i>CARD15</i>	0.981	1.010	0.432-2.360
<i>OCTN</i>	0.028	1.434	1.039-1.980
<i>OCTN</i> (adj <i>CARD15</i> )	0.021	1.479	1.061-2.061
TTCC* <i>CARD15</i>	0.632	0.821	0.367-1.839
ATG* <i>IL23</i> * <i>OCTN</i> * <i>CARD15</i>	0.428	0.651	0.226-1.880

Individual contribution of *CARD15* (at least 1 variant), *ATG16L1* (GG/AG vs AA), *IL23R* rs11209026 (AG/AA vs GG), and *OCTN* dyploype (TTCC) to risk CD. adj *CARD15*: Contribution from the term after adjustment for *CARD15* genotype.

several novel genes and loci involved in the pathogenesis of IBD have been uncovered, as well as the successful identification and replication of multiple susceptibility genes for CD<sup>[11,15,18,20,21,32]</sup>, such as *IL23R* and *ATG16L1*. However, these genetic variations account for only a small portion of the overall genetic susceptibility to CD, and their contribution to the pathogenesis of UC is even lower.

The present study confirms the reported association between *IL23R* and *ATG16L1* variants and susceptibility to CD, both in the case-control and family-based analysis, and in both adult- and pediatric-onset disease. Moreover, we also replicated the significant association between the rs11209026 variant of *IL23R* and UC ( $P = 0.018$ ), suggesting that this gene may also have a role in the genetic susceptibility to UC. Although the size of the cohort was sufficiently large, consisting

of more than 1600 IBD patients, we were unable to detect an association between *IL23R* and *ATG16L1* variants and any sub-phenotypes for both diseases, as described in the majority of published studies, thus suggesting that the overall effect of these variants on certain CD subtypes<sup>[22,23,31,34-36,38]</sup> is much weaker than that observed in *CARD15*. Moreover, we found no statistical evidence for epistatic interaction between *IL23R*, *ATG16L1*, *OCTN1/2*, and *CARD15* genes. Since our cohort also included a large number of pediatric cases (368 diagnosed at age < 19 years), this study confirmed that the rs2241880 polymorphism in the *ATG16L1*<sup>[34,37]</sup> gene and the rs7517847, rs11209026 polymorphisms in the *IL23R* gene<sup>[22,25,27-30]</sup> also influence susceptibility to CD in pediatric-onset patients, at allele and genotype frequencies comparable to that seen in adults.

Recent studies have reported genotype-phenotype associations in adult-pediatric onset CD and *ATG16L1* Ala197Thr variant. Specifically, Prescott *et al*<sup>[34]</sup> demonstrated an association with ileal form of CD with or without colonic involvement (61.7%) but not with isolated colonic disease (52.2%), as well as with diagnosis at an earlier age ( $\leq 16$  years at diagnosis: 63.8%). Subsequently, van Limbergen *et al*<sup>[38]</sup> confirmed the association of isolated ileal disease with rs2241880G-allele ( $P = 0.02$ ) in a combined genotype-phenotype analysis of early and adult onset CD, although after stratifying CD cases in early- and adult-onset the association did not reach statistical significance ( $P = 0.28$  and  $P = 0.08$ , respectively), probably due to the reduced power of the sample sizes.

We observed rs2144880G variant allele frequency of 55% in colonic disease (L2 ± L4), which increased to 60% in pure ileal disease (L1 ± L4), without significant

association with any sub-phenotypes. To definitively answer this question, studies with a larger cohort of CD are needed to increase the statistical power, particularly when considering SNPs that only show a modest increase in the odds ratios for susceptibility. Moreover, different ethnic populations and true heterogeneity may also explain these differences.

IL-23 is a heterodimeric cytokine, composed of the IL-12p40 and p19 subunits. Human and mouse IL-23 share structural homologies with IL-12, and exhibit similar activities, but differ in their capacity to stimulate populations of specific memory T cells, activated to produce the pro-inflammatory mediators IL17 and IL16<sup>[43]</sup>. This so-called TH17 T-cell subset expresses the master transcription factor POU2F1 and mediates chronic inflammatory and autoimmune diseases in animal models<sup>[44]</sup>. IL-23 binds with a complex consisting of IL-23R and IL-12R $\beta$ . IL-23R associates constitutively with Jak2 and in a ligand-dependent manner with stat3. The human IL-23R gene is on human chromosome 1 within 150 kb of IL-12R $\beta$ 2<sup>[45]</sup>. It appears that IL-23 plays a unique role in the initiation and perpetuation of innate and T cell-mediated forms of IBD, and variations in the early IL-12 and IL-23 dependent regulatory mechanisms may impact the subsequent inflammatory response. The strong effect of the protective allele, first identified by Duerr *et al.*<sup>[15]</sup>, could potentially be exploited to define functional outcomes and targeted therapy that to date remain as possibilities.

*ATG16L1* is a member of a large family of genes involved in autophagy, a mechanism by which cells recycle redundant organelles, an essential process in the resistance to pathogens, targeted for immune evasion by viruses and bacteria<sup>[46]</sup>. The exact functional impact of the rs2241880 variant is currently unknown, although the *ATG16L1* protein is a key component of autophagy and the T300A substitution occurs in an evolutionarily conserved domain<sup>[32]</sup>. The gene is expressed in intestinal epithelial cell lines and functional knockdown of this gene abrogates autophagy of *Salmonella typhimurium*<sup>[16]</sup>. The important role of autophagy as predisposing factor to CD has recently been highlighted with the identification of variants in immunity-related guanosine triphosphatase (IRGM) gene, involved in elimination of intracellular bacteria, and susceptibility to CD<sup>[47]</sup>. How this process is implicated in the pathogenesis of CD remains unclear, although it further supports the concept of inflammatory barrier disorder<sup>[48]</sup>.

Our results provide an independent confirmation of the association between the candidate genetic variations in *IL23R* and *ATG16L1* genes and CD, and reinforce the role of these new polymorphisms as genetic determinants in IBD. Further research is needed to understand how *IL23R* and *ATG16L1* variants contribute to disease susceptibility in IBD, and whether they have therapeutic implications.

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## COMMENTS

### Background

It is widely accepted that ulcerative colitis (UC) and Crohn's disease (CD) result from an inappropriate response of a defective mucosal immune system to indigenous flora and other luminal agents in a genetically susceptible host. Eleven inflammatory bowel disease (IBD) genome-wide linkage analyses in families with multiple IBD affected members, and two meta-analyses have identified several linkage regions. Following the identification of *NOD2* (or *CARD15*), the first gene contributing to CD susceptibility (IBD1 locus), further fine mapping studies have identified a risk haplotype (IBD5 locus) on chromosome 5q, and another polymorphism on the *disk large homolog 5* (*DLG5*) gene. However, these risk-associated variants and many others reported with conflicting results, explain only a minority of the genetic risk in IBD. Whole genome association (GWA) studies have rapidly led to the identification of novel susceptibility loci such as *interleukin-23 receptor* (*IL23R*) and *autophagy-related 16-like 1* gene (*ATG16L1*).

### Research frontiers

An uncommon coding mutation (Arg381Gln) in the *IL23R* gene has been identified as conferring strong protection against CD with ileal involvement. Further GWA and replication studies have consistently confirmed strong association between variations at *IL23R* and CD, with a large number of replications in adult- and pediatric-onset cohorts, without significant association with specific CD sub-phenotype. Moreover, an association with UC has also been observed. The non-synonymous (Ala197Thr) variant of *ATG16L1* was found to be associated with CD, but not UC, and has been consistently demonstrated in a number of independent studies in adult subjects, but with conflicting data in pediatric IBD cohorts.

### Innovations and breakthroughs

The present study included a large population of Italian IBD patients (763 CD and 843 UC), including 368 patients with pediatric onset of disease (age at diagnosis < 19 years), 546 healthy parents and 749 healthy controls. All patients were accurately phenotyped. Two SNPs of *IL23R*, one of the *ATG16L1*, and three of the *CARD15* genes were genotyped, and were also evaluated for interaction with specific clinical features among genes.

### Applications

The present study confirms the reported association between *IL23R* and *ATG16L1* variants and susceptibility to CD, both in the case-control and family-based analysis, and both in adult- and pediatric-onset disease. Moreover, we also replicated the significant association of the rs11209026 variant of *IL23R* with UC, suggesting that this gene may also have a role in genetic susceptibility to UC. Although the size of the cohort was large, consisting of more than 1600 IBD patients, we were unable to detect an association between *IL23R* and *ATG16L1* variants and any sub-phenotypes for both diseases.

## Terminology

GWA is the Genome Wide Association which is examined through a chip platform evaluating several thousand SNPs. SNP is Polymorphism of Single Nucleotide evaluated to investigate variants of gene probably modifying the gene function.

## Peer review

This is a nice follow-up study which confirms the association between the candidate genetic variations in *IL23R/ATG16L1* and IBD, in both pediatric- and adult-onset population.

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