REPORT

Genetic Analysis of Innate Immunity in Crohn's Disease and Ulcerative Colitis Identifies Two Susceptibility Loci Harboring *CARD9* and *IL18RAP*

Alexandra Zhernakova,[1] Eleanora M. Festen,[3,4] Lude Franke,[1,4] Gosia Trynka,[4] Cleo C. van Diemen,[4] Alienke J. Monsuur,[1,10] Marianna Bevova,[1] Rian M. Nijmeijer,[2] Ruben van 't Slot,[1] Roel Heijmans,[6] H. Marike Boezen,[5] David A. van Heel,[8] Adriaan A. van Bodegraven,[7] Pieter C.F. Stokkers,[9] Cisca Wijmenga,[1,4,*] J. Bart A. Crusius,[6] and Rinse K. Weersma[3]

The two main phenotypes of inflammatory bowel disease (IBD)—Crohn's disease (CD) and ulcerative colitis (UC)—are chronic intestinal inflammatory disorders with a complex genetic background. Using a three-stage design, we performed a functional candidate-gene analysis of innate immune pathway in IBD. In phase I, we typed 354 SNPs from 85 innate immunity genes in 520 Dutch IBD patients (284 CD, 236 UC) and 808 controls. In phase II, ten autosomal SNPs showing association at $p < 0.006$ in phase I were replicated in a second cohort of 545 IBD patients (326 CD, 219 UC) and 360 controls. In phase III, four SNPs with $p < 0.01$ in the combined phase I and phase II analysis were genotyped in an additional 786 IBD samples (452 CD, 334 UC) and 768 independent controls. Joint analysis of 1851 IBD patients (1062 CD, 789 UC) and 1936 controls demonstrated strong association to the *IL18RAP* rs917997 SNP for both CD and UC (p_{IBD} 1.9×10^{-8}; OR 1.35). Association in CD is independently supported by the Crohn's disease dataset of the Wellcome Trust Case Control Consortium (imputed SNP rs917997, $p = 9.19 \times 10^{-4}$). In addition, an association of the *CARD9* rs10870077 SNP to CD and UC was observed ($p_{IBD} = 3.25 \times 10^{-5}$; OR 1.21). Both genes are located in extended haplotype blocks on 2q11-2q12 and 9q34.3, respectively. Our results indicate two IBD loci and further support the importance of the innate immune system in the predisposition to both CD and UC.

The inflammatory bowel diseases (IBDs) are common, chronic, gastrointestinal inflammatory disorders that comprise two major forms—Crohn's disease (CD [MIM 266600]) and ulcerative colitis (UC [MIM 191390]). Their combined prevalence is estimated at 100–200/100,000 in developed countries.[1,2] The etiology of CD and UC is complex and consists of an impaired immune response to the commensal bacterial flora in a genetically susceptible host.

In 2001 the *NOD2* gene (also known as *CARD15* [MIM 605956]) on chromosome 16 was identified as the first susceptibility gene for CD.[3] *NOD2* is a part of the innate immunity system and belongs to the family of pattern-recognition receptors (PRRs) that recognize bacterial peptidoglycans; it is among one of the first lines of defense against microbial flora in the gastrointestinal tract. Since the discovery of *NOD2* as a CD susceptibility gene, the role of the innate immune system in IBD has been studied extensively, and mutations in a number of genes of the innate immunity pathway have been found to be associated with CD susceptibility.[3–5] The most promising results suggest an association for *NOD1* (MIM 605980) and Toll-like receptor 4 (*TLR4)* (MIM 603030) with CD.[4,6]

Whole-genome association studies performed during recent years have been remarkably successful in providing better insight into the genetic background of CD. Recently described CD susceptibility variants in *IL23R* (MIM 607562), *ATG16L1* (MIM 610767), and *IRGM* (MIM 608212), at 5p13.1 and other loci, have been successfully replicated in multiple independent populations.[7–11] However, all these findings together still only partly explain the genetic background of CD, and many reported associations require further replication and validation. So far, no convincing genetic associations have been established for UC.

In this study, we performed an extensive candidate-pathway genetic screen for IBD susceptibility by using a multistage case-control design. We aimed to investigate the innate immunity pathway, including the pattern-recognition receptor genes (PRRs) and their downstream targets.[12–17] The study included the Toll-like receptor (TLR) pathway with the TLR genes 1 to 10 (MIM 601194, 603028, 603029, 603030, 603031, 605403, 300365, 300366, 605474, and 606270, respectively), molecules important for recognizing lipopolysaccharides by distinct TLRs (*CD14* [MIM 158120] and *LY96* [MIM 605243]), downregulators of TLR activity (*TOLLIP* [MIM 606277] and *PPARG* [MIM 601487] and *SIGIRR* [MIM 605478]), adaptor molecules (*MYD88* [MIM 602170], *TICAM1 (TRIF)* [MIM 607601], *TICAM2* [MIM 608321], and *TIRAP* [MIM 606252]), downstream effectors of *MYD88* (*TRAF6* [MIM 602355], *IRAK2-4* [MIM 603304, 604459, and

[1]Complex Genetics Section, Department of Medical Genetics, [2]Department of Surgery, University Medical Center Utrecht, Utrecht, the Netherlands; [3]Department of Gastroenterology and Hepatology, [4]Department of Genetics, [5]Department of Epidemiology, University Medical Center Groningen and University of Groningen, Groningen, the Netherlands; [6]Department of Pathology, Laboratory for Immunogenetics, [7]Department of Gastroenterology and Hepatology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands; [8]Institute of Cell & Molecular Science, Queen Mary's School of Medicine and Dentistry, London, United Kingdom; [9]Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, the Netherlands
[10]Present address: Netherlands Vaccine Institute, Bilthoven, the Netherlands.
*Correspondence: c.wijmenga@umcutrecht.nl
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606883]), and downstream effectors of *TRIF* (*CFLPR* [MIM 603599], *FADD* [MIM 602457], *TBK1* [MIM 604834], and all 12 components of the NF-κβ pathway). The Toll-independent recognition of pathogen-associated molecular patterns (PAMPs) included genes involved in delivery of muramyl dipeptides into the intracellular space (*PEPT1* [*SLC15A1*] [MIM 600544] and *PEPT2* [*SLC15A2*] [MIM 602339]), downstream pathways of NODs (*APAF1* [MIM 602233], *RIPK2* [MIM 603455], *CARD9* [MIM 607212], and *GRIM19* [MIM 609435]), inhibitors of NOD signaling (*CARD6* [MIM 609986], *CARD7* [MIM 606636], *CARD12* [MIM 606831], *CIAS1* [MIM 606416]), and other genes involved in PAMPs recognition. We also included interferon regulatory factors that are activated by the pathways described above and a number of cytokines and their receptors (*IL-1*, *2*, *12*, *15*, *18*, *23*, and *IFN1A* and *B*) that are important in innate immunity. Two well-described PRR genes—*NOD1* and *NOD2*—have been previously studied in IBD populations and were therefore not included in this study.[18] Some candidate genes involved in the pathway were excluded from our list as a result of a lack of haplotype tagging SNPs in the gene region (e.g., *IRAK1*).

In total, we included 85 genes located in 74 genomic regions in our screen (Table S1 in the Supplemental Data available online). Each investigated locus included the coding part of a gene and at least 3 kb of the promoter region and 2 kb of the 3′ end. The flanking region was extended (to ~20 kb) for ~10% of the genes that are located either in extended haploblocks or in SNP-desert regions. Tag SNPs were selected with the Tagger program and the following parameters: $r^2 > 0.8$, minor allele frequency (MAF) > 0.1, multimarker tagging. SNP genotyping was performed with the GoldenGate assay on an Illumina BeadStation 500GX (Illumina, San Diego, USA). Analysis was performed with Bead Studio software. As quality-control measures, samples showing a mismatch between X chromosome genotypes and stated gender and duplicate samples were removed; genotype call rate and Hardy-Weinberg equilibrium within controls and cases were determined. Only samples with a call rate above 95% were included in further analysis. Initially, 455 tag SNPs were included in this study. A large number of SNP assays failed (n = 101, 22%), in part because of an error in oligonucleotide synthesis at Illumina's manufacturing facility (the company subsequently accepted responsibility and reimbursed us). In addition, we excluded nonpolymorphic and dubiously clustered SNPs (Table S2), resulting in 354 successfully genotyped SNPs that we used for further analysis. Three SNPs showed strong deviation from Hardy-Weinberg equilibrium in the controls ($p_{HWE} < 0.002$) and were discarded from further analysis.

The population studied consisted of 1851 IBD patients (CD 1062, UC 789). IBD patients were recruited from the VU University Medical Center, Amsterdam (n = 520),[19] University Medical Center Groningen (n = 545)[20], and the Academic Medical Center, Amsterdam (n = 786)[21], the Netherlands, and were all of European descent. All patients were diagnosed according to accepted clinical, endoscopic, radiological, and histological criteria[2]; 66.9% of CD patients and 45.8% of UC patients were females. The control group (n = 1936) comprised three independent groups: (i) healthy blood donors from Amsterdam (n = 381) and Utrecht (n = 427), the Netherlands[22]; (ii) healthy spouses of patients included in different, non immune-mediated projects in Utrecht (n = 360)[23], and (iii) 768 controls from the general-population-based Vlagtwedde/Vlaardingen cohort (individuals of Dutch descent, Groningen and Rotterdam area) participating in the last survey in 1989 and 1990.[24,25] Only individuals for whom at least three out of four grandparents were born in the Netherlands were included in this study; 45.6% of controls were females. All patients and controls gave informed consent, and the study was approved by the ethics review committees of each of the participating hospitals. All DNA samples and data in this study were handled anonymously. The design of the study is illustrated in Table 1.

In phase I of the study, 520 IBD patients (284 CD, 236 UC) and 808 controls were successfully genotyped for 354 SNPs. The association was calculated by an allele frequency test (1 df χ^2 test) and by a genotype frequency test (2 df χ^2 test) (columns showing "p-allele" and "p-genotypes" in Table S1). Hardy-Weinberg equilibrium was calculated from a comparison of expected and observed genotypes in 2 × 3 χ^2 tables (columns showing HWE controls and HWE cases in Table S1). From the 74 genomic regions included in the study, the strongest association was observed with the rs7539625 SNP located in *IL23R* [MIM 607562] ($p_{CD} = 2.63 \times 10^{-5}$). The association for the *IL23R* gene with CD has been well established.[7,20,26,27] Association results for all SNPs included in the study are presented in Table S1.

We performed a haplotype analysis on all tested loci, since the selection of tag SNPs was based on a multimarker strategy and in order to recover information that was missed due to failed SNPs. Haplotypes were investigated using the Haploview application.[28] We observed an association with nine genes on a haplotype level at $p < 0.006$, which is the same p-value cut off we chose to use for the single SNP analysis. All associated haplotypes, except one, were located in the genes that also showed association on a single SNP level with the same selected p-value cut-off ($p < 0.006$). One locus – comprising the IL18 receptors on chromosome 2q11-2q12 – was significantly associated in the haplotype analysis, but not at the single SNP level. We observed that the haplotype including the rs2287037*G-rs1035130*G-rs2241116*C and rs6706002*A alleles occurred more frequently in IBD cases (26.1%) than in controls (21.4%) ($p = 0.006$).

We extensively investigated the haplotype structure of the *IL18R1-IL18RAP* receptor locus using the HapMap data.[29] The IBD-associated haplotype is located in a block with strong linkage disequilibrium (LD) expanding ~350 kb. This LD block includes four genes: the 3′ parts of *IL1RL1* (MIM 601203), *IL18R1* (MIM 604494), *IL18RAP*

Table 1. Control and Case Groups Included in the Study

Study Groups	Dutch Cases	Dutch Controls
Phase I: VUMC Amsterdam	IBD n = 520 284 CD 236 UC	Healthy blood donors (n = 808) from Amsterdam (n = 381) and Utrecht (n = 427)
Phase II: UMC Groningen	IBD n = 545 326 CD 219 UC	UMC Utrecht controls (n = 360)
Phase III: AMC Amsterdam	IBD n = 786 452 CD 334 UC	Population controls from Groningen area (n = 768)
Electronic Replication in the Imputed WTCCC Dataset		
WTCCC	IBD n = 1748 1748 CD 0 UC	WTCCC controls (n = 2938)

Three independent case groups were recruited from the VU University Medical Center (VUMC), Amsterdam, University Medical Center (UMC), Groningen, and the Academic Medical Center (AMC), Amsterdam. Imputed WTCCC data are publicly available online.

(MIM 604509) and 5′ part of *SLC9A4* (MIM 600531). Forty-two SNPs have a single allele that is in perfect LD with our associated haplotype in the HapMap data and can therefore tag this haplotype at a single SNP level. None of the 42 SNPs were typed in the current project (Supplementary Table 1). However, one of these SNPs – rs917997 – was tested on the set of Dutch controls for another of our projects. In the 791 controls that were genotyped both for the *IL1RL1-IL18R1-IL18RAP-SLC9A4* haplotypes and for the single SNP rs917997, the haplotype structure was found to be similar to the HapMap data. We observed that the associated haplotype rs2287037*G-rs1035130*G-rs2241116*C-rs6706002*A was in perfect LD with the rs917997*A allele and they are therefore perfect proxies for each other (Table 2). Based on these findings, SNP rs917997 was used for further replication of the *IL1RL1-IL18R1-IL18RAP-SLC9A4* locus.

We performed a replication study of the ten most strongly associated autosomal genes identified by single SNP- or haplotype analysis in either CD, UC or IBD ($p < 0.006$) in phase I. This replication was conducted on a second, independent, IBD cohort (phase II). In genes where two or more SNPs showed association, only the most significantly associated SNP was used for further replication. The *IL23R* gene was not included in the replication since this gene has already been established as a risk factor for IBD in the Dutch IBD cohort.[20]

Nine SNPs were tested in the second IBD cohort, comprising 545 Dutch IBD patients (326 CD and 219 UC) from the University Medical Center Groningen[20] and 360 independent controls (Table 3). Genotyping of all polymorphisms was performed with TaqMan probes and primers, using assays developed by Applied Biosystems, and an ABI 7900HT system (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). To ensure that the annotation of C/G and T/A SNPs both on Taqman and Illumina was performed on the same strand, we did additional TaqMan genotyping of 50 controls for rs8177676 T/A, rs10418239 C/G and rs10870077 C/G. All the called control samples showed 100% concordance of the genotypes obtained from the Taqman and Illumina platforms. The association was calculated by allele frequency test (1 df χ^2 test). All SNPs were in HWE ($p_{HWE} > 0.05$) in control group.

Of the nine SNPs replicated in phase II, rs917997 showed association in both the CD and UC cohorts ($p = 0.0002$; odds ratio [OR] 1.60 [95% CI: 1.25–2.04] and $p = 0.003$; OR 1.52 [95% CI: 1.15 – 2.00], respectively) (Table 3). Combined analysis for rs917997 (either directly genotyped or imputed from other SNPs) of the patient cohorts from phases I and II showed association to the *IL1RL1-IL18R1-IL18RAP-SLC9A4* locus in both CD ($p = 1.20 \times 10^{-5}$) and UC ($p = 4.50 \times 10^{-5}$) (Table 3). After Bonferroni correction for multiple testing of 354 SNPs, the association with rs917997 remained significant. In addition, the combined analysis of phases I and II also showed association with rs842647 in *REL* to UC ($p = 0.003$; OR 0.78 [95% CI: 0.65–0.92]) rs10418239 in *CARD8* to UC ($p = 0.004$; OR 1.26 [95% CI: 1.08–1.48]) and with rs10870077 in *CARD9* to UC ($p = 0.003$; OR 1.26 [95% CI: 1.08–1.47]).

To further confirm the association of rs917997 and the *CARD8*-rs10418239 and *CARD9*-rs10870077 SNPs, we performed a third analysis (phase III) in which these three SNPs were tested in an IBD cohort of 452 CD and 334 UC cases from Amsterdam and 768 population controls from Groningen, the Netherlands.[21] SNPs were genotyped by Taqman as described above. Analysis of the phase III

Table 2. Linkage Disequilibrium between the rs2287037-rs1035130-rs2241116-rs6706002 haplotype block and the rs917997*A Allele in 791 Dutch Control Samples

rs2287037	rs1035130	rs2241116	rs6706002	rs917997	Frequency	Counts
G	G	C	G	G	0.397	626.6: 951.4
A	A	A	A	G	0.219	345.7: 1232.3
G	**G**	**C**	**A**	**A**	**0.213**	**336.1: 1241.9**
A	G	C	G	G	0.089	140.4: 1437.6
A	A	C	A	G	0.076	120.0: 1458.0

A total of 791 controls were typed for rs2287037-rs1035130-rs2241116-rs6706002 haplotypes and for single SNP rs917997 located in the *IL18R1-IL18RAP* gene locus. All haplotypes above a frequency of 0.005 are shown. The rs917997*A allele and rs2287037*G-rs1035130*G-rs2241116*C-rs6706002*A haplotype (in bold) tag the same haplotype and are therefore perfect proxies for each other.

Table 3. Replication of the Ten Associated SNPs from the Phase I Screen in the Phase II Cohort

			Control Phase I	Cases Phase I			Control Phase II	Cases Phase II			Control Phases I and II	Cases Phases I and II		
Gene	**Name**	**Minor Allele**	**MAF**	**MAF**	**P Value**	**OR (95% CI)**	**MAF**	**MAF**	**P Value**	**OR (95% CI)**	**MAF**	**MAF**	**P Value**	**OR (95% CI)**
Crohn's Disease														
IKBKE	rs12728136	A	0.143	0.188	0.010	1.40 (1.09–1.80)	0.178	0.151	0.182	0.82 (0.62–1.10)	0.154	0.169	0.25	1.12 (0.93–1.35)
REL	rs842647	G	0.355	0.300	0.013	0.77 (0.63– 0.94)	0.354	0.365	0.679	1.05 (0.83–1.32)	0.354	0.332	0.189	0.91 (0.78–1.05)
IL18RAP	rs917997[a]	A	0.214	0.252	0.067	1.24 (0.99–1.55)	0.218	0.309	0.0002	1.60 (1.25 –2.04)	0.216	0.282	1.20×10^{-5}	1.43 (1.22–1.68)
CARD6	rs10512747	A	0.157	0.110	0.006	0.67 (0.50–0.90)	0.138	0.146	0.691	1.07 (0.78–1.45)	0.151	0.128	0.07	0.83 (0.67–1.02)
CARD9	rs10870077	G	0.437	0.470	0.172	1.14 (0.94–1.39)	0.455	0.466	0.680	1.05 (0.84–1.3)	0.442	0.468	0.15	1.11 (0.96–1.28)
IL15RA	rs8177676	A	0.088	0.111	0.116	1.30 (0.95–1.77)	0.101	0.1	0.973	1.00 (0.70–1.42)	0.092	0.105	0.22	1.16 (0.92–1.47)
CHUK	rs11597086	C	0.386	0.456	0.004	1.33 (1.10–1.62)	0.419	0.375	0.096	0.83 (0.66–1.03)	0.396	0.414	0.32	1.08 (0.93–1.24)
LGP2	rs12600570	A	0.117	0.130	0.401	1.14 (0.85 –1.52)	0.129	0.118	0.539	0.90 (0.65–1.26)	0.121	0.124	0.78	1.03 (0.84–1.28)
CARD8	rs10418239	G	0.337	0.340	0.912	1.01 (0.83–1.24)	0.349	0.37	0.421	1.10 (0.88–1.37)	0.341	0.356	0.38	1.07 (0.92–1.24)
VISA	rs8116776	A	0.283	0.217	0.002	0.07 (0.56–0.88)	0.266	0.274	0.757	1.04 (0.81–1.33)	0.278	0.246	0.04	0.85 (0.72–1.00)
Ulcerative Colitis														
IKBKE	rs12728136	A	0.143	0.214	0.0002	1.64 (1.26–2.12)	0.178	0.127	0.022	0.68 (0.48–0.95)	0.154	0.173	0.19	1.15 (0.94–1.42)
REL	rs842647	G	0.355	0.302	0.038	0.79 (0.64– 0.99)	0.354	0.292	0.036	0.76 (0.58–0.98)	0.354	0.298	0.003	0.78 (0.65–0.92)
IL18RAP	rs917997*	A	0.214	0.271	0.010	1.37 (1.08–1.73)	0.218	0.298	0.003	1.52 (1.15–2.00)	0.216	0.284	4.50×10^{-5}	1.44 (1.21–1.72)
CARD6	rs10512747	A	0.157	0.146	0.561	0.93 (0.69–1.23)	0.138	0.13	0.697	0.94 (0.66–1.34)	0.151	0.139	0.36	0.91 (0.73–1.13)
CARD9	rs10870077	G	0.437	0.515	0.003	1.37 (1.11–1.68)	0.455	0.483	0.359	1.12 (0.88–1.43)	0.442	0.500	0.003	1.26 (1.08–1.47)
IL15RA	rs8177676	A	0.088	0.131	0.006	1.57 (1.14–2.15)	0.101	0.091	0.597	0.90 (0.60–1.37)	0.092	0.112	0.08	1.25 (0.97–1.61)
CHUK	rs11597086	C	0.386	0.443	0.027	1.26 (1.03–1.55)	0.419	0.392	0.371	0.89 (0.70–1.14)	0.396	0.419	0.24	1.10 (0.94–1.29)
LGP2	rs12600570	A	0.117	0.169	0.003	1.55 (1.17–2.06)	0.129	0.133	0.861	1.04 (0.73–1.48)	0.121	0.152	0.02	1.31 (1.05–1.64)
CARD8	rs10418239	G	0.337	0.411	0.003	1.37 (1.11–1.69)	0.349	0.374	0.360	1.13 (0.87–1.45)	0.341	0.395	0.004	1.26 (1.08–1.48)
VISA	rs8116776	A	0.283	0.233	0.031	0.77 (0.61–0.98)	0.266	0.266	0.995	1.00 (0.76–1.32)	0.278	0.248	0.09	0.86 (0.72–1.03)
Inflammatory Bowel Disease (Crohn's Disease + Ulcerative Colitis)														
IKBKE	rs12728136	A	0.143	0.200	0.0001	1.50 (1.22–1.84)	0.178	0.141	0.038	0.76 (0.59–0.98)	0.154	0.170	0.13	1.13 (0.96–1.33)
REL	rs842647	G	0.355	0.300	0.003	0.78 (0.66 - 0.92)	0.354	0.336	0.439	0.93 (0.75 - 1.12)	0.354	0.317	0.01	0.85 (0.75 - 0.96)
IL18RAP	rs917997*	A	0.214	0.261	0.006	1.29 (1.08–1.55)	0.218	0.304	0.00007	1.56 (1.25–1.95	0.216	0.282	2.82×10^{-7}	1.43 (1.25–1.64)
CARD6	rs10512747	A	0.157	0.126	0.027	0.78 (0.62–0.97)	0.138	0.139	0.931	1.01 (0.77–1.33)	0.151	0.133	0.08	0.86 (0.72–1.02)
CARD9	rs10870077	G	0.437	0.490	0.007	1.24 (1.06–1.45)	0.455	0.474	0.434	1.08 (0.89–1.31)	0.442	0.482	0.008	1.17 (1.04–1.32)
IL15RA	rs8177676	A	0.088	0.120	0.008	1.41 (1.09–1.81)	0.101	0.097	0.781	0.95 (0.69–1.31)	0.092	0.108	0.07	1.20 (0.98–1.46)
CHUK	rs11597086	C	0.386	0.450	0.001	1.30 (1.11–1.52)	0.419	0.383	0.122	0.86 (0.71–1.04)	0.396	0.416	0.18	1.09 (0.96–1.23)
LGP2	rs12600570	A	0.117	0.148	0.020	1.31 (1.05–1.65)	0.129	0.124	0.760	0.95 (0.72–1.27)	0.121	0.136	0.12	1.15 (0.96–1.37)
CARD8	rs10418239	G	0.337	0.372	0.066	1.16 (0.99–1.37)	0.349	0.372	0.329	1.10 (0.90–1.35)	0.341	0.372	0.03	1.15 (1.01–1.30)
VISA	rs8116776	A	0.283	0.224	0.001	0.73 (0.61–0.88)	0.266	0.271	0.818	1.02 (0.83–1.27)	0.278	0.247	0.02	0.85 (0.75–0.98)

Phase I included 520 IBD samples (284 CD, 236 UC) and 808 controls; phase II included 545 IBD samples (326 CD, 219 UC) and 360 controls; the combined analysis of phase I and II includes 1065 IBD samples (610 CD, 455 UC) and 1168 controls. Allele call for C/G and T/A SNPs is annotated according to the TaqMan genotype calls.

[a] rs917997 was either genotyped directly or imputed from rs2287037*G-rs1035130*G-rs2241116*C-rs6706002*A haplotype (in the phase I IBD cases).

data confirmed the association of the *IL18RAP* SNP rs917997 in CD ($p = 0.012$; OR 1.27 [95% CI (1.05–1.54)]) and showed a similar trend in UC ($p = 0.091$; OR 1.20 [95% CI (0.97–1.48)]).Association of the *CARD9* SNP rs10870077 was also confirmed in the phase III CD and UC groups (Table 4). However, association of the *CARD8* SNP rs10418239 and the *REL* – rs842647 was not observed in the phase III cohort, although it remained moderately significant in the combined UC (*CARD8*) and UC and CD (*REL*) dataset.

The combined analysis of 1851 IBD patients included in phases I, II, and III (1062 CD and 789 UC samples; 1936 controls) showed strong association to rs917997 of the *IL1RL1-IL18R1-IL18RAP-SLC9A4* locus with both CD ($p = 6.3 \times 10^{-7}$; OR 1.36; 95% CI [1.21–1.54]) and UC ($p = 2.46 \times 10^{-5}$; OR 1.34; 95% CI [1.17–1.53]). SNP rs10870077 in the *CARD9* locus showed stronger association with UC ($p = 4.03 \times 10^{-5}$; OR 1.28; 95% CI [1.14–1.44]) and moderate association with CD ($p = 0.0057$; OR 1.16; 95% CI [1.04–1.3]). Association of rs917997 with both CD and UC, and association of rs10870077 with both the UC and IBD groups, remained significant after Bonferroni correction for the total number of 354 SNPs included in phase I. Associations in the combined dataset for *CARD8* and *REL* genes were not significant after correction for multiple testing.

In this study we performed a candidate screen of innate immunity pathway genes in IBD and observed a strong association with a common SNP located in the *IL1RL1-IL18R1-IL18RAP-SLC9A4* locus on chromosome 2q11-2q12. Interestingly, association of the same allele of the *IL18RAP* rs917997 gene with celiac disease (MIM 212750; a distinct disorder but one that is also characterized by intestinal inflammation) has been reported in a recent GWAS study[22] and replicated in an independent cohort of celiac-disease patients from three different populations.[30]

Replication of genetic findings in multiple populations is an important step in establishing a genetic effect on disease predisposition. Recently a large genome-wide association study was performed by the Wellcome Trust Case Control Consortium (WTCCC). This study included 2,000 CD patients and 3,000 controls from the UK.[8] We investigated publicly available WTCCC results for association of *IL1RL1-IL18R1-IL18RAP-SLC9A4* (rs917997) and *CARD9* (rs10870077) in the UK CD cohort (WTCCC data accessed on November 21, 2007). Neither SNP had been typed on the Affymetrix platform, so only imputed genotypes were available.[31] In the WTCCC CD dataset, the rs917997 showed moderate association with the same allele ($p = 9.19 \times 10^{-4}$) (Table 5). The combined Cochran-Mantel-Haenszel test of allelic association of rs917997 with CD in the Dutch and UK (WTCCC) data resulted in an even stronger association of this SNP with CD (p-value ($p_{M\text{-}H} = 8.63 \times 10^{-9}$).

To ensure that association in the 2q11-2q12 region is limited to the *IL1RL1-IL18R1-IL18RAP-SLC9A4* locus, we investigated the region of ~898 kb (Chr2:102040449–102947000) by including this and the neighboring LD blocks from the GWAS WTCCC CD dataset. Among 238 SNPs that have been genotyped in the ~898 kb block, the strongest association was observed with three SNPs, all of which are located within the *IL18RAP* gene (Figure S1). This strongly indicates that in the WTCCC data association in CD is limited to the *IL1RL1-IL18R1-IL18RAP-SLC9A4* block itself, rather than being a side effect of association in the neighboring haplotype blocks.

Because of strong LD, fine mapping within the *IL1RL1-IL18R1-IL18RAP-SLC9A4* locus is not possible by genetic means. The ~350 kb LD block includes four genes, two of which, *IL18RAP* and *IL18R1*, are receptors for the IL18 protein and were selected for our candidate-gene study. Remarkably, a *cis* effect of the associated rs917997 on the level of gene expression of the IL18RAP transcript was recently observed in the celiac-disease study.[30] This observation prioritizes the *IL18RAP* gene among its neighbors as a candidate gene for IBD. The role of the IL18 protein in CD is now being increasingly recognized. IL18 is mainly produced by antigen-presenting cells and stimulates the production of interferon-γ and pro-inflammatory cytokines such as TNF-α and IL-1β.[32] Expression of IL18 is increased in mucosal biopsies from IBD patients compared to controls, and in *involved* versus *non-involved* lesions.[33–35] Furthermore, IL18 mRNA levels are upregulated in intestinal epithelial cells and lamina propria mononuclear cells in CD patients, and the bioactive form of IL18 could only be found in patients but not in controls.[35,36] Finally, several mouse models for CD show that blocking IL18 with an IL18-binding protein attenuates the intestinal inflammation.[37,38] The IL18 receptor complex is expressed on intestinal epithelial cells and consists of the ligand-binding chain (IL18R1, IL18Rα) and a signal-transducing chain (IL18RAP, IL18Rβ).[39] IL18 receptors, in response to IL18, activate the NF-κβ pathway, which is a central element in the pathogenesis of CD.[2,33] The *IL1RL1-IL18R1-IL18RAP-SLC9A4* locus is therefore a very likely candidate for IBD pathogenesis. Interestingly, SNPs in the *IL18* gene also tended to be associated with IBD in our initial screen ($p = 0.0086$, Table S1), although the association was only moderate and needs further replication.

Another locus that shows association predominantly with UC is located on 9q34.3 and includes the *CARD9* gene, which encodes an adaptor molecule of PRR signaling. *CARD9* is an attractive candidate gene for IBD association because it is essential in the process of stimulating the innate immune signaling by intracellular and extracellular pathogens.[40] Three studies of CARD9-deficient mice showed that CARD9 is required for inducing the cytokine response and for protection from fungal and bacterial infection.[41–44] Upon stimulation by intracellular pathogens, CARD9 specifically interacts with NOD2, which leads to increased cytokine production via activation of MAPK.[43] Alternatively, stimulation of other PPR family molecules by extracellular pathogens leads to NF-κβ activation through the syk-CARD9 interaction.[45,46] One possible

Table 4. Association of *REL*-rs842647, *IL18RAP*-rs917997, *CARD9*-rs10870077, and *CARD8*-rs10418239 SNPs in the Phase III IBD Cohort and in the Combined Dataset

			Control Phase III	Cases Phase III versus Control Phase III			Control Phases I, II, and III	Cases Phases I, II, and III versus Controls Phases I, II, and III		
Name	**SNP**	**Minor Allele**	**MAF**	**MAF**	**P Value**	**OR (95% CI)**	**MAF**	**MAF**	**P Value**	**OR (95% CI)**
Crohn's Disease										
rs842647	A; G	G	0.357	0.320	0.067	0.85 (0.71–1.01)	0.355	0.327	0.028	0.88 (0.79–0.99)
rs917997	G; A	A	0.235	0.281	0.012	1.27 (1.05–1.54)	0.223	0.282	6.31×10^{-7}	1.36 (1.21–1.54)
rs10870077	C; G	G	0.425	0.480	0.0093	1.25 (1.06–1.48)	0.436	0.473	5.70×10^{-3}	1.16 (1.04–1.30)
rs10418239	C; G	G	0.367	0.354	0.534	0.95 (0.80–1.13)	0.351	0.355	0.759	1.02 (0.91–1.14)
Ulcerative Colitis										
rs842647	A; G	G	0.357	0.339	0.402	0.93 (0.76–1.12)	0.355	0.315	0.005	0.83 (0.74–0.94)
rs917997	G; A	A	0.235	0.269	0.091	1.20 (0.97–1.48)	0.223	0.278	2.46×10^{-5}	1.34 (1.17–1.53)
rs10870077	C; G	G	0.425	0.494	0.0034	1.32 (1.10–1.59)	0.436	0.497	4.03×10^{-5}	1.28 (1.14–1.44)
rs10418239	C; G	G	0.367	0.366	0.95	0.99 (0.82–1.21)	0.351	0.383	0.030	1.15 (1.01–1.30)
Inflammatory Bowel Disease (Crohn's Disease + Ulcerative Colitis)										
rs842647	A; G	G	0.357	0.328	0.090	0.88 (0.76–1.02)	0.355	0.322	0.002	0.86 (0.78–0.95)
rs917997	G; A	A	0.235	0.276	0.009	1.24 (1.05–1.46)	0.223	0.28	1.90×10^{-8}	1.35 (1.22–1.50)
rs10870077	C; G	G	0.425	0.486	0.00081	1.28 (1.11–1.48)	0.436	0.484	3.25×10^{-5}	1.21 (1.11–1.33)
rs10418239	C; G	G	0.367	0.359	0.649	0.97 (0.83–1.12)	0.351	0.366	0.166	1.07 (0.97–1.18)

Phase III included 786 IBD cases (452 CD, 334 UC) and 768 controls. The combined analysis included 1851 IBD cases (1062 CD; 789 UC) and 1936 controls.

Table 5. Association of rs917997 and rs10870077 with Crohn's Disease in the WTCCC Data

SNP	Quality Prediction	MAF Controls	MAF CD Cases	P Value	OR (95% CI)
rs10870077	0.8752	0.4172	0.4570	0.0002	1.18 (1.08–1.28)
rs917997	0.9993	0.2185	0.2483	0.0009	1.18 (1.07–1.30)

mechanism for CARD9's involvement in IBD pathogenesis is via regulation of the IL17 response to the pathogens. Activation of the syk-CARD9 complex via dectin-1 (the C-type lectin molecule) promotes the maturation of dendritic cells, production of pro-inflammatory cytokines, including IL23, and differentiation of T cells to T_H-17 cells. The process of differentiation of T_H-17 cells under *Candida albicans* infection is markedly reduced in CARD9$^{-/-}$ cells.[44] Interestingly, the current hypothesis on the role of the recently described *IL23R* gene in IBD pathogenesis also suggests an effect from *IL23R* mutations on T_H-17 cell development.[47,48] It is possible that mutations in *CARD9* and *IL23R* share a similar pathogenesis mechanism for affecting the disease susceptibility.

A recent expression study on 400 blood samples of asthma patients indicated a strong *cis* effect from a perfect proxy of the *CARD9* rs10870077 (rs4077515, $r^2 = 1$ in CEU HapMap samples, and $r^2 = 0.95$ in Dutch controls) on expression of CARD9 (effect rs4077515*G allele 0.538, H2: 14.17, p value 1.1×10^{-13}) ("mRNA by SNP browser").[33,49,50] This suggests that the rs10870077 genotype probably has a *cis* functional implication on CARD9 signaling via modification of CARD9 expression.

In the WTCCC dataset, the *CARD9* rs10870077 SNP was less efficiently imputed (average maximum posterior call = 0.87), probably as a result of the location of the gene on the distal part of chromosome 9 and low coverage of this region by the genotyping platform. However, the imputed results show an increased frequency of the minor rs10870077*G allele in CD cases (45.7%), compared to controls (41.7%) ($p = 1.75 \times 10^{-4}$), which is consistent with our observations for CD (Table 5). The combined Cochran-Mantel-Haenszel test of CARD9 rs10870077 SNP with CD in the Dutch and WTCCC datasets indicates moderate but consistent association to CD in both populations (p value ($p_{M\text{-}H} = 1.20 \times 10^{-6}$). In our dataset, the *CARD9* gene showed stronger association to UC than to CD. Further replication of these data in international IBD cohorts is necessary for estimating the effect of *CARD9* locus polymorphisms on CD and UC.

In this study we selected the *CARD9* as a functional candidate gene; however *CARD9* is located in an extended haplotype block spanning ~120 kb and also including the following genes: *GPSM1* (MIM 609491), *LOC728489* (*DNLZ*), *SNAPC4* (MIM 602777), *SDCCAG3*, *PMPCA*, *INPP5E*, and *KIAA0310* (*SEC16A*). The strong LD within the block does not allow us to exclude other genes in the region from association with IBD, and moreover, poor coverage of this locus in the WTCCC dataset does not allow to exclude the neighboring blocks (only 18 SNPs have been typed in the ~778 kb block including the *CARD9* locus and neighboring blocks in the WTCCC dataset). A number of genes located in the *CARD9* block are also attractive functional candidates for association with IBD. The first one to mention, the *GPSM1* gene belongs to activators of G protein signaling and is known to play a regulatory role in autophagosome formation in intestinal cells.[51] Recent GWAS studies discovered the two autophagy genes, *ATG16L1* and *IRGM*, and highlighted the important role of the autophagy pathway in the pathogenesis of CD.[52] Another plausible functional candidate, *SDCCAG3* (serologically defined colon cancer antigen 3), is involved in regulating expression of the tumor necrosis factor (TNF) receptor on the cell surface and in the anti-apoptotic mechanism during TNF signaling. TNF is a widely expressed cytokine involved in inflammation and innate immunity.[53] Further investigation of the *CARD9* and surrounding genes is needed to verify the true causative gene in the 9q34.3 locus.

In this study we performed a comprehensive analysis of candidate genes from 85 genes in the innate-immunity molecular pathway, in a group of 1851 IBD patients and 1936 controls, and discovered the IBD-susceptibility gene locus that includes the *IL1RL1-IL18R1-IL18RAP-SLC9A4* genes on chromosome 2q11-2q12. We also observed association of the *CARD9* variant, located in extended haploblock on 9q34.3, predominantly with UC, although this latter finding needs further fine mapping and confirmation.

Supplemental Data

One figure and two tables are available with this article online at http://www.ajhg.org/.

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Web Resources

URLs for data presented here are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim

HapMap, http://www.hapmap.org, for information on SNPs and linkage disequilibrium in population controls

Tagger, http://www.broad.mit.edu/mpg/tagger/, or selection of tagging SNPs

mRNA by SNP browser, http://www.sph.umich.edu/csg/liang/asthma/, database that contains information on correlation of SNP genotypes and gene expression

WTCCC summary statistics, http://www.wtccc.org.uk/info/summary_stats.shtml, for data on Crohn's disease cases and control genotypes for rs10870077 and rs917997 from the Wellcome Trust Case Control Consortium dataset

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