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• *RAPID COMMUNICATION* •

Combined carriership of *TLR9* -1237C and *CD14* -260T alleles enhances the risk of developing chronic relapsing pouchitis

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CONCLUSION: There is no evidence that the SNPs predispose to the need for IPAA surgery. The significant increase of the combined carriership of the *CD14* -260T and *TLR9* -1237C alleles in the chronic relapsing pouchitis group suggests that these markers identify a subgroup of IPAA patients with a risk of developing chronic or refractory pouchitis.

Key words: Pouchitis; Innate immunity; Single nucleotide polymorphisms; *CD14*; *TLR9*

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Abstract

AIM: To investigate the single nucleotide polymorphisms (SNPs) in genes involved in bacterial recognition and the susceptibility to pouchitis or pouchitis severity.

METHODS: Analyses of *CD14* -260C>T, *CARD15/NOD2* 3020insC, Toll-like receptor (*TLR*)4 +896A>G, *TLR9* -1237T>C, *TLR9*+2848G>A, and *IRAKM* +22148G>A SNPs were performed in 157 ileal-pouch anal anastomosis (IPAA) patients (79 patients who did not develop pouchitis, 43 infrequent pouchitis patients, 35 chronic relapsing pouchitis patients) and 224 Italian Caucasian healthy controls.

RESULTS: No significant differences were found in SNP frequencies between controls and IPAA patients. However, a significant difference in carriership frequency of the *TLR9*-1237C allele was found between the infrequent pouchitis and chronic relapsing pouchitis groups [$P = 0.028$, odd's ratio (OR) = 3.2, 95%CI = 1.2-8.6]. This allele uniquely represented a 4-locus *TLR9* haplotype comprising both studied *TLR9* SNPs in Caucasians. Carrier trait analysis revealed an enhanced combined carriership of the alleles *TLR9* -1237C and *CD14* -260T in the chronic relapsing pouchitis and infrequent pouchitis group ($P = 0.018$, OR = 4.1, 95%CI = 1.4 -12.3).

INTRODUCTION

Patients with ulcerative colitis may need surgery for their disease and proctocolectomy with ileal-pouch anal anastomosis (IPAA) is the surgical procedure of choice for the management of these patients[1,2]. Most patients undergoing IPAA for severe colitis or chronic continuous disease achieve good functional results, but some patients develop pouchitis, a non-specific idiopathic inflammation of the ileal reservoir. Frequency rates of pouchitis are highly variable, ranging 10-59% depending on the length of follow-up and the diagnostic criteria used[3]. Though the origin of pouchitis remains unknown, genetic and immunological factors are likely to be involved in addition to ileal mucosa that needs to adapt to its new role as a reservoir[4]. This is illustrated by the fact that pouchitis occurs almost exclusively in patients with IPAA for ulcerative colitis and not in patients with IPAA for familial adenomatous polyposis, a hereditary non-inflammatory disease of the colon with high risk of developing colon cancer. An important role of luminal bacteria in the development of pouchitis is underscored by various reports on bacterial overgrowth and dysbiosis in pouchitis[5] and is further confirmed by the efficacy of antibiotic and probiotic therapy[6,7].

Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), are essential components of the innate

immune system as recognition of microbial products occurs via PRRs that are expressed by innate effector cells. Microbial recognition results in a rapid and efficient immune response against invading microorganisms[8].

Given the role of luminal bacteria in driving the inflammatory response in pouchitis, identification and functional characterization of polymorphisms in innate immunity genes may provide insight in a possible genetically determined susceptibility to pouchitis and/or chronic relapsing pouchitis[9].

CD14 is part of the endotoxin/lipopolysaccharide (LPS) receptor complex[8] and is important in conjunction with TLR4 and with TLR2[10] in the recognition of LPS, a membrane glycolipid on Gram-negative bacteria. *CD14* may also recognize cell membrane components of Gram-positive mycobacteria and viruses[11-15]. *CD14* exists in a membrane form on monocytes and neutrophils and in a soluble form in serum[16-18]. The SNP at position -260C>T (also known as *CD14*-159C>T) in the promoter region of the *CD14* gene (located on chromosome 5q31) is associated with enhanced transcriptional activity[19] and significantly higher *CD14* serum levels[20]. Increased expression of *CD14* in macrophages has been found in inflammatory bowel disease (IBD)[21]. An association of the *CD14*-260C>T gene polymorphism with IBD[22,23] and atherosclerosis[24] has been described. Genetically determined variation in *CD14* serum levels may have functional consequences given the ability of soluble *CD14* to confer pathogen responsiveness to cells such as intestinal, epithelial and endothelial cells that do not express *CD14* on their membranes[25].

The TLR4 gene is located on chromosome 9q32-q33. The TLR4+896A>G SNP affects the leucine-rich repeat domain of TLR4 and is associated with hyporesponsiveness to LPS[26] with increased susceptibility to severe bacterial infections and IBD[27] and may predispose to septic shock with Gram-negative microorganisms[28,29].

TLR9 is required for the recognition of CpG motifs, short sequences of unmethylated DNA predominantly present in bacterial DNA. CpG motifs have immunostimulatory activity by inducing dendritic cell maturation, B-cell proliferation and production of cytokines, including interleukin-6 (IL-6) and interleukin-12 (IL-12)[30,31]. *TLR9* signaling has been shown to mediate the resolution of intestinal inflammation in experimental colitis[32], suggesting that the release of bacterial DNA from the microflora might favor immune homeostasis. The promoter *TLR9* -1237T>C SNP located on chromosome 3p21.3 is associated with susceptibility to asthma in European Americans, but not in Hispanic or African Americans[33] and the marker D3S1076 in this region shows association with IBD in a classical TDT test[42]. Török and colleagues[34] studied the *TLR9* -1237T>C and *TLR9*+2848 G>A SNPs in German patients with Crohn's disease, ulcerative colitis and healthy blood controls and found that the allele *TLR9* -1237C carrier status is associated with Crohn's disease compared to controls. *TLR9* +2848 G>A allele frequencies are not different between the study groups.

CARD15/NOD2 is a cytoplasmatic bacterial PRR expressed in monocytes and intestinal epithelial cells and mediates response against muramyl dipeptide derived from peptidoglycan[35,36]. The CARD15/NOD2 gene is located on chromosome 16q12. Three major polymorphisms in this gene (R702W, G908R, and L1007 frameshift mutation) are associated with susceptibility to Crohn's disease, possibly as a result of a defective response against muramyl dipeptide derived from peptidoglycan[37]. Recently, an increased frequency of the L1007 frameshift mutation has been observed in Italian patients with ulcerative colitis when compared to controls[38].

The intracellular domains of TLRs are homologous to the interleukin-1 receptor (IL-1R) type I intracellular domain and use a common pathway of intracellular signaling with shared components including the protein kinase IL-1R-associated kinase1 (IRAK1) and IRAK-M, as a negative regulator of TLR signaling. Cytokine production increases in IRAK-M-/- macrophages after TLR/IL-1 stimulation and bacterial challenge, while endotoxin tolerance reduces in these cells. Furthermore, IRAK-M-/- mice have increased inflammatory responses to bacterial infection and develop intestinal inflammation. These data suggest that IRAK-M has a regulatory function in TLR/IL-1R signaling and innate immune homeostasis[39]. The *IRAKM* (or *IRAK3*) gene is located at chromosome 12q14.2 within the IBD2 region associated with ulcerative colitis[40,41]. Genetic variation in the *IRAKM* gene may be involved in the development of chronic intestinal inflammation. For this reason, we chose to analyze a non-synonymous SNP in exon 5 resulting in an Ile/Val substitution.

A candidate gene approach, based on the determination of frequencies of functional SNPs, can be used to investigate the relevance of genes to the disease susceptibility and severity. Carrier trait analysis investigates combinations of SNPs and allows studying the implication of different SNPs in disease susceptibility and severity as a result of their synergistic action.

The aim of this study was whether SNPs in innate immunity genes could contribute to the susceptibility to pouchitis and/or severity of pouchitis. We chose candidate genes of *CD14, TLR4, TLR9, NOD2/CARD15*, and *IRAKM* for their involvement in bacterial recognition and intracellular signaling pathways.

MATERIALS AND METHODS

Patients

One hundred and fifty-seven unrelated patients with IPAA for ulcerative colitis and 224 healthy blood donors were studied. All individuals were Italian Caucasians. Consent was obtained and the local ethics committee approved the protocol. Their demographic and clinical information is described in Table 1. IPAA patients were subdivided into three test groups according to the pouchitis pattern. One group consisted of IPAA patients who had never developed pouchitis, another group consisted of IPAA patients who had up to two episodes of pouchitis during IPAA (infrequent pouchitis) and third group consisted of IPAA patients who developed three or more episodes of

pouchitis (chronic relapsing pouchitis).

DNA isolation

Venous blood (5-10 mL) was drawn and genomic DNA was isolated using standard protocols. Polymorphisms of the *CD14, TLR4, TLR9, CARD15/NOD2*, and *IRAKM* genes in these three groups were analyzed. Genomic DNA (5-100 ng) was used for each genotyping.

Analysis of gene polymorphisms

Polymerase chain reaction (PCR) for RFLP analyses was performed on a thermal cycler GeneAmp 9700 (Perkin-Elmer Cetus, Norwalk, CT, USA). Digested fragments were analyzed on a 4% agarose gel except for the *IRAKM* SNP analyzed on a 2% agarose gel and visualized with an UV-illuminator after ethidium bromide staining. SNPs were analyzed with the TaqMan assay (Applied Biosystems, Foster City, CA, USA). MGB TaqMan probes and primer pairs were designed with Primer Express software (version 2.0). TaqMan thermocycling consisted of an initial step at 50 ℃ for 2 min and denaturation at 95 ℃ for 10 min followed by 40 cycles of denaturation at 95 ℃ for 15 s and annealing/extension at 60 ℃ for 1 min. We used the ABI Prism 7000 sequence detector (Applied Biosystems) for data acquisition.

Genotyping

CD14-260C>T genotyping (NCBI SNP CLUSTER ID: rs2569190) was performed by PCR. Primers used were: forward primer 5'-TCA CCT CCC CAC CTC TCT T-3' and reverse primer 5'-CCT GCA GAA TCC TTC CTG TT-3'. The PCR conditions were initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 59 ℃ for 30 s, extension at 72 ℃ for 1 min and a final extension at 72 ℃ for 5 min followed by cooling to 4 ℃. The 107-bp amplicons were digested overnight with *HaeIII* (New England Biolabs, UK). Digestion resulted in two fragments of 83 and 24 bp (C allele) or 107 bp (T allele), respectively.

Genotyping of the TLR4+896 A>G SNP (NCBI SNP CLUSTER ID: rs4986790) was performed with forward primer 5'-TTT ACC CTT TCA ATA GTC ACA CTC A-3' and reverse primer 5'-AGC ATA CTT AGA CTA CTA CCT CCA TG-3'. PCR conditions were: initial denaturation at 94 ℃ for 5 min followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, extension at 72 ℃ for 30 s and a final extension at 72 ℃ for 5 min followed by cooling to 4 ℃. The 102-bp amplicons were digested overnight with *NcoI* (New England Biolabs, UK). Digestion resulted in two fragments of 80 and 22 bp (G allele) or 102 bp (A allele), respectively.

CARD15/NOD2 3020InsC (*CARD15 L1007fs*) (NCBI SNP CLUSTER ID: rs2066847) genotyping was performed with forward primer 5'-GGC AGA AGC CCT CCT GCA GGG CC-3' and reverse primer 5'-CCT CAA AAT TCT GCC ATT CC-3'. PCR conditions were: initial denaturation at 94 ℃ for 5 min followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 59 ℃ for 30 s, extension at 72 ℃ for 45 s and a final extension at 72 ℃ for 5 min followed by cooling to 4 ℃. The 150-bp amplicons were digested overnight with *ApaI*. Digestion resulted in a fragment of 150 bp (no insertion) or 128 bp and 22 bp (insertion C), respectively.

TLR9-1237T>C (NCBI SNP CLUSTER ID: rs5743836) genotyping was performed with TaqMan method. Primers used were: forward primer 5'-GGC CTT GGG ATG TGC TGT T-3' and reverse primer 5'-GGT GAC ATG GGA GCA GAG ACA-3'. Dual-labeled fluorogenic hybridization MGB-probes used were: CTGCCTGAAAACT 5' Fluor Label (FAM, 6-carboxyfluorescein) and CTGGAAACTCCCC 5' Fluor Label (VIC).

TLR9+2848G>A genotyping (NCBI SNP CLUSTER ID: rs352140) was performed with TaqMan method. Primers used were: forward primer 5'-CCG GTC TGC AGG TGC TAG AC-3' and reverse primer 5'-CCA AAG GGC TGG CTG TTG TA-3'. Dual-labeled fluorogenic hybridization MGB probes used were: AGCTACCGCGACTGG 5' Fluor Label (FAM) and AGCTACCACGACTGGA 5' Fluor Label (VIC).

Genotyping of the *IRAKM*+22148G>A exon 5 SNP (NCBI SNP CLUSTER ID: rs1152888) was performed by PCR with forward primer 5´-AGT GGA AC T GAT GTC CTG TGA CAG-3´ and reverse primer 5´-GCA ACA CAT TGA CCT AAT GAC CAG-3´.

The PCR conditions were: initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 50 s, at 60 ℃ for 50 s, at 72 ℃ for 150 s and a final extension at 72 ℃ for 5 min followed by cooling to 4 ℃. Digestion overnight with RsaI (Invitrogen Life Technologies) of the 505-bp amplicons resulted in two fragments of 188+317 bp (allele G) or 505 bp (allele A).

Table 1 Demographic features of IPAA patients and healthy controls

	IPAA patients	Healthy controls
Total number (*n*)	157	224
Gender M/F	88/69	118/106
Mean age in yr (SD)	42.9 (11.8)	45.8 (12.8)
Range	17-73	21-77
Median	41	45.5

Statistical analysis

Hardy-Weinberg equilibrium was determined in healthy controls to assess the Mendelian inheritance. Comparisons of the genotypes between control and different groups of IPAA patients were performed by Fisher's exact or χ^2 two-tailed tests where appropriate. Carrier trait analysis was performed to determine whether combinations of SNPs were acting synergistically on the risk of developing pouchitis or of predisposing to chronic relapsing pouchitis. Adjusted odd's ratio (OR) and 95% confidence intervals (95%CI) were calculated. $P<0.05$ was considered statistically significant.

RESULTS

Characteristics of patients and control groups

The demographic features of IPAA patients and healthy

Table 2 Clinical data obtained from IPAA patients

IPAA patients	Mean (SD)	Median	Range
Age (yr) at diagnosis of ulcerative colitis	29.7 (11.7)	27	8-61
Time (yr) from diagnosis of ulcerative colitis to IPAA surgery	6.3 (5.5)	5	0-34
Time from IPAA surgery to first episode of pouchitis			
Total pouchitis group (n = 78)	2.8 (3.1)	2	0-12
Infrequent pouchitis (≤2 episodes, n = 43)	3.7 (3.3)	3	0-12
Chronic relapsing pouchitis (≥3 episodes, n = 35)	2 (2.6)	1	0-12
Pattern of pouchitis			
Duration of pouch (yr)			
No episodes of pouchitis (n = 79)	6.3 (4.1)	5	0-14
Infrequent pouchitis (≤2 episodes, n = 43)	7.6 (3.8)	7	1-16
Chronic relapsing pouchitis (≥3 episodes, n = 35)	7.9 (3.6)	7	2–14

Table 3 Genotypes of the *CD14, CARD15, TLR4, TLR9*, and *IRAKM* gene polymorphisms in subgroups of patients with IPAA and controls

Polymorphisms	Genotype	Controls n = 224 (%)	No pouchitis n = 79 (%)	Infrequent pouchitis n = 43 (%)	Chronic relapsing pouchitis n = 35 (%)
CD14 –260 C>T	CC	55 (24.6)	24 (30.4)	11 (25.6)	7 (20)
	CT	102 (45.5)	34 (43)	21 (48.8)	22 (62.9)
	TT	67 (29.9)	21 (26.6)	11 (25.6)	6 (17.1)
CARD15 3020InsC	WT/WT	218 (97.3)	77 (97.5)	43 (100)	34 (97.1)
	WT/InsC	6 (2.7)	2 (2.5)	0 (0)	1 (2.9)
	InsC/InsC	0 (0)	0 (0)	0 (0)	0 (0)
TLR4 +896 A>G	AA	208 (92.9)	73 (92.4)	38 (88.4)	33 (94.3)
	AG	16 (7.1)	5 (6.3)	5 (11.6)	2 (5.7)
	GG	0 (0)	1 (1.3)	0 (0)	0 (0)
TLR9 –1237 T>C	TT	158 (70.5)	52 (65.8)	34 (79.1)	19 (54.3)
	TC	60 (26.8)	24 (30.4)	9 (20.9)	14 (40)
	CC	6 (2.7)	3 (3.8)	0 (0)	2 (5.7)
TLR9 +2848 G>A	GG	40 (17.9)	20 (25.3)	9 (20.9)	6 (17.1)
	GA	104 (46.4)	38 (48.1)	22 (51.2)	16 (45.7)
	AA	80 (35.7)	21 (26.6)	12 (27.9)	13 (37.2)
IRAKM +22148G>A	GG	181 (80.8)	64 (81)	33 (76.7)	28 (80)
	GA	42 (18.8)	14 (17.7)	7 (16.3)	7 (20)
	AA	1 (0.4)	1 (1.3)	3 (7)	0 (0)

controls are shown in Table 1. No statistical differences were found in the variables including gender and age between the IPAA group and healthy controls.

The clinical characteristics of patients with IPAA for ulcerative colitis were summarized. Information on the pattern of pouchitis within the group of IPAA patients for ulcerative colitis (patients who did not develop a pouchitis, patients who had infrequent pouchitis and patients who suffered from chronic relapsing pouchitis, respectively) is shown in Table 2. No statistically significant differences were found in gender and age between controls and IPAA patients as well as in the duration of pouch and the first episode of pouchitis after IPAA, respectively and among IPAA groups and between infrequent pouchitis and chronic relapsing pouchitis groups.

Genotyping

The genotype frequencies in the control group were in Hardy-Weinberg equilibrium for the *CD14, TLR4, TLR9, CARD15/NOD2,* and *IRAKM* gene polymorphisms. The genotype frequencies of these polymorphisms are described in Table 3. No significant differences in allele-, genotype- or carrier frequencies of the gene polymorphisms were found between the healthy controls and IPAA patients.

No significant differences in allele-, genotype- or carrier frequencies of the gene polymorphisms were found between the three subgroups of IPAA patients except that the carriership of allele *TLR9* -1237 C was more frequent in patients with chronic relapsing pouchitis (45.7%) as compared to those with infrequent pouchitis (20.9%) (P =0.028, OR = 3.2, 95%CI = 1.2-8.6). When the combined groups of infrequent pouchitis and chronic relapsing pouchitis (i.e. total pouchitis group) were compared to the patients without pouchitis, carriership of this allele was not significantly different (P = 0.87, OR = 1.1, 95%CI = 0.6-2.1).

TLR9 haplotype

The two analyzed *TLR9* SNPs were chosen based on the study of Lazarus *et al*.[33] in which a set of four frequent *TLR9* SNPs designated as *TLR9* -1486, *TLR9* -1237, *TLR9* +1174, and *TLR9* +2848, were described. Genotyping of both *TLR9* -1237 and *TLR9* +2848 could distinguish all four-locus haplotypes commonly present in the European American population. We therefore calculated the haplotypic genotypes in Italian Caucasians (Table 4). The haplotype frequencies in the healthy controls were identical to the European-Americans as reported by Lazarus *et al*.[33].

Table 4 Frequencies of *TLR9* haplotypes formed by -1237 T>C and +2848 G>A SNPs

TLR9 -1237	TLR9 +2848	Haplotype	Controls	No pouchitis	Infrequent pouchitis	Chronic relapsing pouchitis
			2*n* = 448	2*n* = 158	2*n* = 86	2*n* = 70
T	G	I	181 (40)	51 (32)	40 (47)	28 (40)
T	A	II	195 (44)	77 (49)	37 (43)	24 (34)
C	A	III	69 (15)	29 (18)	9 (10)[1]	18 (26)[1]
C	G	IV	3 (1)	1 (1)	0	0

[1]*P* = 0.018, OR = 3.0, 95%CI = 1.2-7.1.

Haplotype III was more frequent in chronic relapsing pouchitis as compared to infrequent pouchitis (*P* = 0.018; OR = 3.0, 95%CI = 1.2-7.1). This haplotype, however, did not show nucleotides uniquely present (tag SNPs) on position -1486 (allele T present in haplotypes I and III) or on position +1174 (allele G present in haplotypes II and III), indicating that allele *TLR9*-1237C could provide the strongest association.

Carrier trait analysis

To investigate if SNPs in different genes could act synergistically on disease susceptibility and/or severity, carrier trait analysis with the associated *TLR9* allele was performed. Simultaneous carriership of alleles *TLR9* -1237C and *CD14* -260T was more frequent in patients with chronic relapsing pouchitis as compared to those with infrequent pouchitis (*P* = 0.018, OR = 4.1, 95%CI = 1.4-12.3), which was more significant as compared to the analysis of allele *TLR9* -1237C alone (*P* = 0.028, OR = 3.2, 95%CI = 1.2-8.6). No other significant carrier traits were observed.

DISCUSSION

There is convincing evidence that enteric bacteria play a role in driving the inflammatory response in IBD and that genetic factors contribute not only to the pathogenesis but also to the course and extent of these disorders. Given these processes, we investigated whether polymorphisms in the following genes encoding for proteins involved in innate immunity, *TLR4* +896 A>G, *TLR9* +2848 G>A, *TLR9* -1237T>C, *CD14* -260C>T, *CARD15/NOD2* 3020insC, and *IRAKM* +22148 G>A, were associated with the development of pouchitis, disease frequency or severity.

Analysis of the three subgroups of IPAA patients (i.e. patients who never developed pouchitis, patients with infrequent pouchitis and patients with a chronic refractory form of pouchitis) revealed a positive association of allele *TLR9* -1237C with the risk of developing chronic refractory pouchitis, once these patients developed pouchitis. Haplotype analysis showed that out of the four SNPs defining *TLR9* haplotypes, this allele was uniquely responsible for this finding. Subsequently, we investigated whether interactions of allele *TLR9* -1237C with SNPs in the other candidate genes might strengthen this association. Carrier trait analysis revealed that an even stronger association was apparent with the combination of alleles *TLR9* -1237C and *CD14* -260T. These data suggest that this combination of alleles might be a valuable genetic marker to identify a clinical subgroup of IPAA patients with an enhanced risk of developing chronic pouchitis, compared to alleles of the SNPs *TLR4* +896 A>G, *TLR9* +2848 G>A, *CARD15/NOD2* 3020insC, and *IRAKM* +22148 G>A.

It cannot be excluded that the group of patients who did not develop pouchitis consisted of a mixture of patients who never developed pouchitis on the one hand and patients who proceeded to the infrequent or chronic relapsing pouchitis group on the other hand. This could explain why we did not detect an association of allele *TLR9* -1237C between the no-pouchitis and the chronic relapsing pouchitis groups. It should be noted that no significant differences were found in the mean duration of IPAA between the three groups.

At present it is unknown as to what effect of the *TLR9*-1237 T>C SNP exerts on the expression of *TLR9* given its location in the far promoter region where no DNA-binding site for known transcription factors is apparent. The association observed might therefore result from linkage disequilibrium with another polymorphism(s) in a nearby gene.

The mechanism underlying increased risk of developing chronic relapsing pouchitis by a combined carriership of alleles *TLR9* -1237C and *CD14* -260T might be a dysfunction in bacterial recognition or a lack of an adequate immune response to bacterial challenge. This could start at the level of the plasmacytoid dendritic cells, which play a central role in bacterial recognition, selectively express *TLR9* and have soluble *CD14* facilitating reactivity to a broad array of bacterial components[43] or at the level of the intestinal epithelium. Soluble *CD14* might confer epithelial cell responsiveness[25].

The regulatory role of dendritic cells is of particular importance in the intestine where the mucosal immune system is closely associated with the external environment[44]. Dendritic cells sample bacterial products either indirectly via M cells or directly by reaching between epithelial cells into the gut lumen[45]. In this perspective, it is noteworthy to mention a recent article that reported a lack of immature blood dendritic cells, which possibly migrate to the gut in IBD patients with active disease[46].

Recently, carriership of the *TLR9* -1237C allele has been associated with Crohn's disease[34]. Ileal involvement is present in about 60% of patients with Crohn's disease. Since the pouch is an ileal reservoir that is more vulnerable to the continuous contact with high bacterial titers, it could

be hypothesized that carriership of the allele *TLR9* -1237C (with or without *CD14* -260T) is associated with an impaired immune response at the level of the ileal tissue. Paneth cells are located in the crypts and are central in host defense to luminal bacteria by releasing antimicrobial substances[47,48].

If this is true, carriership of allele *TLR9* -1237C may become an important predictive marker to the enhanced risk of developing refractory chronic pouchitis and eventually pouch failure.

SNPs in different genes might work synergistically and constitute a small to moderate relative risk of developing diseases. Though the observations we described in this article were based on a relatively less number of patients, it should be realized that this study might represent one of the largest series available.

In conclusion, our data suggest that the alleles *TLR9*-1237C and *CD14*-260T synergistically enhance the risk of developing chronic relapsing pouchitis and eventually pouch failure in ulcerative colitis patients who need surgical intervention. Larger studies are required to determine whether this allelic combination becomes a valuable predictive marker and functional studies on the biological role of *TLR9* and *CD14* in pouchitis.

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REFERENCES

1 **Pemberton JH,** Kelly KA, Beart RW Jr, Dozois RR, Wolff BG, Ilstrup DM. Ileal pouch-anal anastomosis for chronic ulcerative colitis. Long-term results. *Ann Surg* 1987; **206:** 504-513
2 **Nicholls RJ,** Moskowitz RL, Shepherd NA. Restorative proctocolectomy with ileal reservoir. *Br J Surg* 1985; **72 Suppl:** S76-579
3 **Gionchetti P,** Amadini C, Rizzello F, Venturi A, Poggioli G, Campieri M. Probiotics for the treatment of postoperative complications following intestinal surgery. *Best Pract Res Clin Gastroenterol* 2003; **17:** 821-831
4 **Sandborn WJ,** Tremaine WJ, Batts KP, Pemberton JH, Phillips SF. Pouchitis after ileal pouch-anal anastomosis: a Pouchitis Disease Activity Index. *Mayo Clin Proc* 1994; **69:** 409-415
5 **Ruseler-van Embden JG,** Schouten WR, van Lieshout LM. Pouchitis: result of microbial imbalance? *Gut* 1994; **35:** 658-664
6 **Gionchetti P,** Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, Poggioli G, Miglioli M, Campieri M. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* 2000; **119:** 305-309
7 **Mimura T,** Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 2004; **53:** 108-114
8 **Wright SD,** Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; **249:** 1431-1433
9 **Shen B,** Lashner B. Can we immunogenotypically and immunophenotypically profile patients who are at risk for pouchitis? *Am J Gastroenterol* 2004; **99:** 442-444
10 **Medzhitov R,** Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; **388:** 394-397
11 **Cleveland MG,** Gorham JD, Murphy TL, Tuomanen E, Murphy KM. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. I*nfect Immun* 1996; ***64:*** 1906-1912
12 **Dobrovolskaia MA,** Vogel SN. Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect* 2002; **4:** 903-914
13 **Kurt-Jones EA,** Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, Finberg RW. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 2000; **1:** 398-401
14 **Vignal C,** Guerardel Y, Kremer L, Masson M, Legrand D, Mazurier J, Elass E. Lipomannans, but not lipoarabinomannans, purified from Mycobacterium chelonae and Mycobacterium kansasii induce TNF-alpha and IL-8 secretion by a *CD14*-toll-like receptor 2-dependent mechanism. *J Immunol* 2003; **171:** 2014-2023
15 **Compton T,** Kurt-Jones EA, Boehme KW, Belko J, Latz E, Golenbock DT, Finberg RW. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J Virol* 2003; **77:** 4588-4596
16 **Frey EA,** Miller DS, Jahr TG, Sundan A, Bazil V, Espevik T, Finlay BB, Wright SD. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J Exp Med* 1992; **176:** 1665-1671
17 **Hailman E,** Vasselon T, Kelley M, Busse LA, Hu MC, Lichenstein HS, Detmers PA, Wright SD. Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. *J Immunol* 1996; **156:** 4384-4390
18 **Landmann R,** Knopf HP, Link S, Sansano S, Schumann R, Zimmerli W. Human monocyte CD14 is upregulated by lipopolysaccharide. *Infect Immun* 1996; **64:** 1762-1769
19 **LeVan TD,** Bloom JW, Bailey TJ, Karp CL, Halonen M, Martinez FD, Vercelli D. A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. *J Immunol* 2001; **167:** 5838-5844
20 **Baldini M,** Lohman IC, Halonen M, Erickson RP, Holt PG, Martinez FD. A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am J Respir Cell Mol Biol* 1999; **20:** 976-983
21 ***Grimm MC,*** Pavli P, Van de Pol E, Doe WF. Evidence for a CD14+ population of monocytes in inflammatory bowel disease mucosa--implications for pathogenesis. *Clin Exp Immunol* 1995; **100:** 291-297
22 **Obana N,** Takahashi S, Kinouchi Y, Negoro K, Takagi S, Hiwatashi N, Shimosegawa T. Ulcerative colitis is associated with a promoter polymorphism of lipopolysaccharide receptor gene, CD14. *Scand J Gastroenterol* 2002; **37:** 699-704
23 **Klein W,** Tromm A, Griga T, Fricke H, Folwaczny C, Hocke M, Eitner K, Marx M, Duerig N, Epplen JT. A polymorphism in the CD14 gene is associated with Crohn disease. *Scand J Gastroenterol* 2002; **37:** 189-191
24 **Hubacek JA,** Rothe G, Pit'ha J, Skodova Z, Stanek V, Poledne R, Schmitz G. C(-260)-->T polymorphism in the promoter of the CD14 monocyte receptor gene as a risk factor for myocardial infarction. *Circulation* 1999; **99:** 3218-3220

25 **Vercelli D.** Learning from discrepancies: CD14 polymorphisms, atopy and the endotoxin switch. *Clin Exp Allergy* 2003; **33:** 153-155

26 **Arbour NC,** Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000; **25:** 187-191

27 **Franchimont D,** Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, Quertinmont E, Abramowicz M, Van Gossum A, Deviere J, Rutgeerts P. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* 2004; **53:** 987-992

28 **Agnese DM,** Calvano JE, Hahm SJ, Coyle SM, Corbett SA, Calvano SE, Lowry SF. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J Infect Dis* 2002; **186:** 1522-1525

29 **Lorenz E,** Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Arch Intern Med* 2002; ***162:*** 1028-1032

30 **Krieg AM,** Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995; **374:** 546-549

31 **Klinman DM,** Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci USA* 1996; **93:** 2879-8283

32 **Rachmilewitz D,** Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, Raz E. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 2004; **126:** 520-528

33 **Lazarus R,** Klimecki WT, Raby BA, Vercelli D, Palmer LJ, Kwiatkowski DJ, Silverman EK, Martinez F, Weiss ST. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (*TLR9*): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. *Genomics* 2003; **81:** 85-91

34 **Torok HP,** Glas J, Tonenchi L, Bruennler G, Folwaczny M, Folwaczny C. Crohn's disease is associated with a toll-like receptor-9 polymorphism. *Gastroenterology* 2004; **127:** 365-366

35 **Inohara N,** Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, Foster SJ, Moran AP, Fernandez-Luna JL, Nunez G. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 2003; **278:** 5509-5512

36 **Girardin SE,** Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 2003; **278**: 8869-8872

37 **Bonen DK,** Cho JH. The genetics of inflammatory bowel disease. *Gastroenterology* 2003; **124:** 521-536

38 **Andriulli A**, Annese V, Latiano A, Palmieri O, Fortina P, Ardizzone S, Cottone M, D'Inca R, Riegler G. The frame-shift mutation of the NOD2/CARD15 gene is significantly increased in ulcerative colitis: an *IG-IBD study. *Gastroenterology* 2004; **126:** 625-627

39 **Kobayashi K,** Hernandez LD, Galan JE, Janeway CA Jr, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 2002; **110:** 191-202

40 **Hampe J,** Schreiber S, Shaw SH, Lau KF, Bridger S, Macpherson AJ, Cardon LR, Sakul H, Harris TJ, Buckler A, Hall J, Stokkers P, van Deventer SJ, Nurnberg P, Mirza MM, Lee JC, Lennard-Jones JE, Mathew CG, Curran ME. A genomewide analysis provides evidence for novel linkages in inflammatory bowel disease in a large European cohort. *Am J Hum Genet* 1999; **64:** 808-816

41 **Duerr RH,** Barmada MM, Zhang L, Davis S, Preston RA, Chensny LJ, Brown JL, Ehrlich GD, Weeks DE, Aston CE. Linkage and association between inflammatory bowel disease and a locus on chromosome 12. *Am J Hum Genet* 1998; **63:** 95-100

42 **Hampe J,** Lynch NJ, Daniels S, Bridger S, Macpherson AJ, Stokkers P, Forbes A, Lennard-Jones JE, Mathew CG, Curran ME, Schreiber S. Fine mapping of the chromosome 3p susceptibility locus in inflammatory bowel disease. *Gut* 2001; **48:** 191-197

43 **Rothenfusser S,** Tuma E, Endres S, Hartmann G. Plasmacytoid dendritic cells: the key to CpG. *Hum Immunol* 2002; **63:** 1111-1119

44 **Stagg AJ,** Hart AL, Knight SC, Kamm MA. The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria. *Gut* 2003; **52:** 1522-1529

45 **Rescigno M,** Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial m`onolayers to sample bacteria. *Nat Immunol* 2001; **2:** 361-367

46 **Baumgart DC,** Metzke D, Schmitz J, Scheffold A, Sturm A, Wiedenmann B, Dignass AU. Patients with active inflammatory bowel disease lack immature peripheral blood plasmacytoid and myeloid dendritic cells. *Gut* 2005; **54:** 228-236

47 **Rumio C, Besusso D,** Palazzo M, Selleri S, Sfondrini L, Dubini F, Menard S, Balsari A. Degranulation of paneth cells via toll-like receptor 9. *Am J Pathol* 2004; **165:** 373-381

48 **Ogura Y,** Lala S, Xin W, Smith E, Dowds TA, Chen FF, Zimmermann E, Tretiakova M, Cho JH, Hart J, Greenson JK, Keshav S, Nunez G. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 2003; **52:** 1591-1597

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