Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms: New data and a meta-analysis

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Abstract

Background: The pathogenesis of inflammatory bowel disease (IBD) involves interactions between the host genetic susceptibility, intestinal microflora and mucosal immune responses through the pattern recognition receptor. Polymorphisms in toll-like receptor 4 (TLR4) induce an aberrant immune response to indigenous intestinal flora, which might favor IBD development. In this study, we aimed to determine whether TLR4 gene was associated with Crohn’s disease (CD) and ulcerative colitis (UC) among Moroccan patients, and evaluated its correlation with clinical manifestation of the disease.

Methods: The study population comprised 117 patients with IBD and 112 healthy unrelated blood donors. TLR4 polymorphisms: Asp299Gly and Thr399Ile were genotyped by polymerase chain reaction-restriction fragment length polymorphism. PCR products were cleaved with Nco I for the Asp299Gly polymorphism and Hinf I for the Thr399Ile polymorphism. Meta-analysis was performed to test the association of 299Gly and 399Ileu carriage with CD, UC and the overall IBD risk.

Results: Our study revealed that the frequency of Asp299Gly and Thr399Ile did not differ significantly between patients and controls in the Moroccan population. However, meta-analysis demonstrated significantly higher frequencies of both Asp299Gly and Thr399Ile SNP in IBD and CD and for 399Ileu carriage in UC patients.

Conclusion: The meta-analysis provides evidence that TLR4 polymorphisms confer a significant increased risk for the overall IBD development.

Keywords: Toll-like receptor 4, Inflammatory bowel disease, Moroccan patients

Background

Inflammatory bowel disease (IBD) is an idiopathic and chronic multifactorial disease of the gastrointestinal tract. Although the precise etiology of IBD is unclear, several factors that play a crucial role in disease pathogenesis such as commensal bacterial flora and genes related to the host immune response have been identified [1,2].

Toll-like receptors are pattern recognition receptors through which host recognizes microbial conserved molecular motifs that are broadly shared by pathogens, therefore they are very important for the regulation of mucosal innate immune responses to intestinal microbes. Perturbations in individual TLR biological signaling can prime to a number of different outcomes and elucidate a system of regulation within the intestine in which each TLR plays a largely non-redundant role in mucosal immunity.

TLR4 gene, the first mammalian TLR identified is located on the long arm of human chromosome 9q32-33 [3]. It encodes the transmembrane receptor that initiates the innate immune response to common gram-negative bacteria [4,5].

TLR4 is the major transducer of lipopolysaccharide (LPS) and binds specifically lipid A moiety. Signal transduction through TLR4 in combination with CD14, and MD-2 leads to activation of the nuclear factor-kB (NF-kB) system through the MyD88-dependent and MyD88-independent pathways and subsequent expression of inflammatory genes encoding cytokines and cell conjugation molecules as part of host defense mechanisms [6-9].

Under healthy conditions TLR4 is only minimally expressed in lamina propria mononuclear cells (LPMNCs)
and intestinal epithelial cells which are partly tolerant to LPS, thus preventing an exaggerated immune response mediated by the large number of bacteria in the intestinal lumen and maintaining a basal state of activation [10,11].

However, TLR4 expression is upregulated in human IBD colitis, maximizing responsiveness to the environment and reflecting an aberrant state of activation [12-15]. Higher levels of TLR4 mRNA and protein were found in the inflamed colonic mucosa in pediatric IBD patients [16]. Two common co-segregating polymorphisms affecting the extracellular domain of the TLR4 (Asp299Gly and Thr399Ile) have been described in humans. Individuals heterozygous for these mutations have a blunted response to inhaled LPS [17]. These polymorphisms are thought to be associated with increased susceptibility to IBD. However, population studies reveal discordant results in geographical distribution.

Thereby, we sought to investigate whether Asp299Gly and Thr399Ile single nucleotide polymorphisms of the gene encoding the TLR4 determine susceptibility to IBD in Moroccan patients and assessed their influence on phenotype expression.

**Methods**

**Study population**

Enrolled in this study were 117 IBD Moroccan patients (83 CD; 34 UC) and 112 healthy unrelated blood donors. The diagnosis of CD or UC was established according to conventional clinical, endoscopic, radiological and histological criteria as previously reported [18,19]. CD was classified according to the Montreal classification [20]. The case report form included questions on disease phenotype and location, age at diagnosis, toxic behavior and other clinical features. The ethics committee of the

**Table 1** Pooled analysis of studies exploring the role of TLR4 Asp299Gly in CD

| Study                | Cases (Events/Total) | Controls (Events/Total) | Odds ratio | 95% CI       | P value |
|----------------------|----------------------|-------------------------|------------|-------------|---------|
| Arnott et al. 2004 [31] | 50/468               | 33/378                  | 1.25       | 0.79 to 1.98|         |
| Franchimont et al. 2004 [32] | 73/668               | 14/278                  | 2.31       | 1.28 to 4.17|         |
| Franchimont et al. 2004 [32] | 26/226               | 14/278                  | 2.45       | 1.25 to 4.81|         |
| Torok et al. 2004 [33] | 14/204               | 12/290                  | 1.70       | 0.77 to 3.77|         |
| Braat et al. 2005 [34] | 68/822               | 13/274                  | 1.81       | 0.98 to 3.33|         |
| Brand et al. 2005 [22] | 29/408               | 15/398                  | 1.95       | 1.03 to 3.70|         |
| Lakatos et al. 2005 [35] | 104/1054             | 48/400                  | 0.80       | 0.56 to 1.15|         |
| Gazouli et al. 2005 [36] | 19/240               | 6/200                   | 2.78       | 1.09 to 7.10|         |
| Oostenbrug et al. 2005 [37] | 53/786              | 27/592                  | 1.51       | 0.94 to 2.43|         |
| Ouburg et al. 2005 [38] | 23/224               | 18/340                  | 2.04       | 1.08 to 3.88|         |
| Fries et al. 2005 [39] | 2/46                 | 2/118                   | 2.63       | 0.36 to 19.29|         |
| Fries et al. 2005 [39] | 10/120               | 2/118                   | 5.27       | 1.13 to 24.60|         |
| Zouiten-Mekki et al. 2009 [40] | 12/180           | 9/160                   | 1.19       | 0.49 to 2.92|         |
| Hong et al. 2007 [41] | 26/364               | 32/376                  | 0.82       | 0.48 to 1.42|         |
| Baumgart et al. 2007 [21] | 6/288               | 16/404                  | 0.51       | 0.20 to 1.33|         |
| Baumgart et al. 2007 [21] | 28/482               | 49/806                  | 0.95       | 0.59 to 1.54|         |
| Browning et al. 2007 [42] | 50/778              | 44/832                  | 1.23       | 0.81 to 1.86|         |
| De Ridder et al. 2007 [23] | 11/144              | 20/488                  | 1.93       | 0.91 to 4.14|         |
| De Ridder et al. 2007 [23] | 63/756              | 20/488                  | 2.12       | 1.27 to 3.56|         |
| Riis et al. 2007 [43] | 32/422               | 152/1236                | 0.58       | 0.39 to 0.87|         |
| Hume et al. 2008 [44] | 87/1238              | 36/720                  | 1.43       | 0.96 to 2.14|         |
| Rigoli et al. 2008 [45] | 10/266               | 8/206                   | 0.96       | 0.38 to 2.49|         |
| Manolakis et al. 2013 [46] | 20/326             | 33/548                  | 1.02       | 0.58 to 1.81|         |
| Current study 2014 | 9/166                | 10/224                  | 1.22       | 0.49 to 3.09|         |
| Total (fixed effects) | 825/10676            | 633/10152               | 1.26       | 1.13 to 1.42| 0.0001 |
| Total (random effects) | 825/10676            | 633/10152               | 1.35       | 1.12 to 1.64|         |
Faculty of Medicine and Pharmacy of Casablanca approved the study and a written informed consent was obtained from all human subjects.

**Molecular analysis of TLR4 polymorphisms**

Genomic DNA was extracted from peripheral blood leukocytes using the salting out procedure. Two single nucleotide variations, corresponding to two amino acid polymorphisms for TLR4, were analyzed: the Asp299Gly (896A/G), rs4986790 and the Thr399Ile (1196C/T), rs4986791.

Typing of the polymorphisms was performed using polymerase chain reaction (PCR) restriction fragment length polymorphism analysis (RFLP).

Upstream and downstream primers used for the PCR amplification were:

F: (5′- AGCATACTTAGACTACTACCTCCATG-3′),
R: (5′- GAGAGATTTGAGTTTCAATGTGGG-3′) for TLR4Asp299Gly
And F: (5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′),
R: (5′-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3′) for TLR4Thr399Ile.

Reactions were performed in a 25 μl volume containing 200 μM of each dNTP (0.5 μl of dNTP mix, 10 mM each), 0.2 μM of each of the forward and reverse primers (0.5 μl of each 10 μM primers), 2 mM MgCl2 (1 μl of MgCl2, 50 mM) and 1 U of Taq DNA polymerase (1 μl of 1U/μl enzyme), 1 PCR buffer (2.5 μl of 10 PCR buffer).

PCR conditions comprised 5 min at 95°C then 35 cycles of denaturing were performed at 95°C for 30 s, annealing at 55°C (Asp299Gly) and at 53°C (Thr399Ile) for 1 min, 72°C for 30 s. A final extension phase of 72°C for 10 minutes was used.

PCR products were cleaved overnight at 37°C with Nco I for Asp299Gly polymorphism and HinfI for Thr399Ile polymorphism (Biolabs). The digests were run on a 3% agarose gel and visualized under UV light using ethidium bromide.

The mutant alleles (GG)/(TT) contained an Nco I/Hinf I restriction site for the Asp299Gly/Thr399Ile polymorphisms respectively, allowing RFLP analysis of the digested products. Digestion at the Nco I site yields fragments of 168 and 20 bp, the one at Hinf I site yields fragments of 98 and 26 bp. The wild-type allele for both polymorphisms remained uncut.

**Statistical analysis**

The data were analyzed with MedCalc 11.6. Chi-square test was used to compare the allele and genotype frequencies between disease and control groups. The Fisher’s exact test was used when appropriate. The observed genotype frequencies were compared with the predicted frequencies by the Hardy Weinberg equilibrium.

The average age was determined by the rank sum test. Associations between genotypes and risk of IBD were

| Study                     | Cases (Events/Total) | Controls (Events/Total) | Odds ratio | 95% CI      | P value |
|---------------------------|----------------------|-------------------------|------------|-------------|---------|
| Arnott et al. 2004 [31]   | 35/492               | 33/378                  | 0.801      | 0.49 to 1.31|         |
| Franchimont et al. (1) 2004 [32] | 32/326             | 14/278                  | 2.052      | 1.07 to 3.93|         |
| Torok et al. 2004 [33]    | 18/196               | 12/290                  | 2.343      | 1.10 to 4.98|         |
| Braat et al. 2005 [34]    | 24/452               | 13/274                  | 1.126      | 0.56 to 2.25|         |
| Gazouli et al. 2005 [36]  | 6/170                | 6/200                   | 1.183      | 0.37 to 3.73|         |
| Oostenbrug et al. 2005 [37] | 21/358              | 27/592                  | 1.304      | 0.72 to 2.34|         |
| Baumgart et al. 2007 [21] (1) | 8/236              | 16/404                  | 0.851      | 0.36 to 2.02|         |
| Baumgart et al. 2007 [21] (2) | 24/290             | 49/806                  | 1.394      | 0.84 to 2.31|         |
| Browning et al. 2007 [42] | 51/810              | 44/832                  | 1.203      | 0.79 to 1.82|         |
| Riis et al. 2007 [43]     | 53/808               | 152/1236                | 0.501      | 0.36 to 0.69|         |
| De Ridder et al. 2007 [23] (1) | 33/452             | 20/488                  | 1.843      | 1.04 to 3.26|         |
| De Ridder et al. 2007 [23] (2) | 4/62               | 20/488                  | 1.614      | 0.53 to 4.88|         |
| Rigoli et al. 2008 [45]   | 3/90                 | 8/206                   | 0.853      | 0.22 to 3.29|         |
| Manolakis et al. 2013 [46] | 41/374              | 33/548                  | 1.921      | 1.19 to 3.10|         |
| Current study 2014        | 6/68                 | 10/224                  | 2.071      | 0.72 to 5.92|         |
| **Total (fixed effects)** | 359/5184            | 457/7244                | 1.092      | 0.94 to 1.26| 0.20    |
| **Total (random effects)** | 359/5184            | 457/7244                | 1.268      | 0.95 to 1.69|         |
estimated by calculating odds ratio (OR) with confidence interval of 95% (CI). P values less than 0.05 were considered significant in disease risk association tests. The χ² test or Fisher test was used to correlate the TLR4 polymorphisms and clinical parameters. The Bonferroni correction method was applied for correction for multiple testing in sub-phenotype analysis; The phenotype genotype correlation was considered statistically significant if the p value was less than 0.005 for CD and 0.007 for UC. According to Power Calculator for Genetic Studies 2006 software (http://www.sph.umich.edu/csg/abecasis/CaTS), this study had 15% of power to detect an OR of 1.5.

**TLR4 meta-analysis**

**Inclusion and exclusion criteria**

Genetic association studies were included in our meta-analysis if they met the following criteria:

(a) Studies that evaluated the association between the TLR4 Asp299Gly, Thr399Ile polymorphisms and IBD, (b) A case control study design, (c) The study reported sufficient data to calculate allele frequencies, odds ratios and confidence intervals of cases and controls for carriage of the TLR4 299Gly and 399Ile alleles.

While major exclusion criteria were: (a) case-only study and review articles (b) absence of the mutant allele in both cases and controls, (c) studies without the raw data of the TLR4 Asp299Gly and Thr399Ile genotypes.

**Pooled studies for case control meta-analysis**

Twelve case-control studies were identified through the literature search.

**Asp299Gly polymorphism**: According to the inclusion criteria, twenty studies were retrieved in CD meta-analysis (Table 1), four of them contained more than one cohort [21-24]. UC meta-analysis reported data from 13 of the included studies (Table 2); two of them

| Table 3 | Pooled analysis of studies exploring the role of TLR4 Thr399Ile in CD |
|---------|---------------------------------------------------------------|
| Study   | Sample size | Cases (Events/Total) | Controls (Events/Total) | Odds ratio | 95% CI   | P value |
| Torok et al. 2004 [33] | CD: 102 | 16/204 | 12/290 | 1.972 | 0.91 to 4.26 |
| | HC: 145 | | | |
| Braat et al. 2005 [34] | CD: 204 | 30/408 | 19/398 | 1.583 | 0.87 to 2.86 |
| | HC: 199 | | | |
| Gazouli et al. 2005 [36] | CD: 120 | 1/240 | 2/200 | 0.414 | 0.037 to 4.60 |
| | HC: 100 | | | |
| Oostenbrug et al. 2005 [37] | CD: 393 | 69/1008 | 29/598 | 1.442 | 0.92 to 2.25 |
| | HC: 296 | | | |
| Zouiten-Mekki et al. 2009 [40] | CD: 90 | 13/180 | 8/160 | 1.479 | 0.59 to 3.66 |
| | HC: 80 | | | |
| Hong et al. 2007 [41] | CD: 182 | 30/364 | 32/376 | 0.966 | 0.57 to 1.62 |
| | HC: 188 | | | |
| Browning et al. 2007 [42] | CD: 389 | 47/778 | 46/832 | 1.099 | 0.72 to 1.67 |
| | HC: 416 | | | |
| De Ridder et al. 2007 [23] | CD: 450 | 72/900 | 22/488 | 1.842 | 1.12 to 3.00 |
| | HC: 244 | | | |
| Rigoli et al. 2008 [45] | CD: 133 | 8/266 | 6/206 | 1.034 | 0.35 to 3.02 |
| | HC: 103 | | | |
| Azzam et al. 2012 [47] | CD: 46 | 26/92 | 22/100 | 1.397 | 0.72 to 2.69 |
| | HC: 50 | | | |
| Manolakis et al. 2013 [46] | CD: 163 | 20/326 | 33/548 | 1.020 | 0.57 to 1.80 |
| | HC: 274 | | | |
| Our study 2014 | CD: 83 | 7/166 | 3/224 | 3.243 | 0.82 to 12.73 |
| | HC: 112 | | | |

**Total (fixed effects)** 339/4932 234/4420 1.345 1.12 to 1.60 0.002

**Total (random effects)** 339/4932 234/4420 1.336 1.11 to 1.59
contained more than one cohort [21,22]. Six studies met one of the exclusion criteria [25-30].

**Thr399Ile Polymorphism:** twelve studies comprising 2466 cases and 2210 controls were included in CD meta-analysis (Table 3), and nine of them in UC meta-analysis with 1358 cases and 1773 controls (Table 4). A meta-analysis combining CD and UC patients for the two tested SNPs: Asp299Gly and Thr399Ile included 13 and 9 studies respectively (Tables 5 and 6).

The risk of IBD associated with the TLR4 polymorphism was estimated for each study by odds ratio (OR) and 95% confidence interval (95% CI). The meta-ORs were estimated using a fixed-effects model with the wild-type allele as reference group. Genetic heterogeneity was tested by Cochran’s (Q) test, I² statistics was used to quantify the between-study heterogeneity effect. When a significant Q test (Q > 0.10; I² > 50%) indicated heterogeneity across studies, data were recombined using a random-effects model to estimate common ORs. The meta-analyses were conducted by Review Manager 5.0 and MedCalc bvba 12.3.0 softwares.

### Results

Hundred and seventeen patients with IBD (83 CD; 34 UC) and 112 control subjects from the general population were genotyped for the presence of TLR4Asp299Gly and Thr399Ile polymorphisms.

The average age of CD, UC patients and controls was 27.6 ± 2.3, 40 ± 5.0 and 31.3 ± 2.1 years respectively. The distributions of genotype and allele frequencies of both TLR4Asp299Gly and Thr399Ile polymorphisms in CD patients (X² = 0.03, P = 0.86; X² = 0.02, P = 0.90) and healthy controls (X² = 2.86, P = 0.24; X² = 0.01, P = 0.94) were in Hardy-Weinberg equilibrium. In patients with UC, genotype and allele frequencies distributions for Asp299Gly polymorphism (X² = 0.03, P = 0.86) were in Hardy-Weinberg equilibrium but not for Th399ILeu polymorphism (X² = 19.05, P < 0.001).

In order to study associations of TLR4 variants in IBD overall and in CD and UC in particular, the distribution of TLR4 polymorphic alleles was assessed. Genotype and allele frequencies are given in Table 7 and genotypic and allelic odds ratios and test P-values are presented in Table 8. None CD nor UC colitis patients were homozygous for G allele. Mutant allele frequency was 5.4% in CD, 8.8% in UC and 4.5% in HC. No significant difference was noticed in allele distributions of the Asp299Gly polymorphism between the control and patient groups. Likewise, no significant association of IBD with the Thr399Ile polymorphism was found in either cohort (allele frequencies: HC 1.3%, CD...
4.2%, UC 4.4%). TT genotype was not observed in both CD patients and HC and only one individual carried the 399Ile variant at both alleles in UC. Co-segregation of TLR4 polymorphic alleles was observed in only 33% of controls (3 out of 9), 33% in UC (2 out of 6) and 60% in CD (6 out of 10).

Meta-analysis of our dataset with the published studies showed a significant association between TLR4 Asp299Gly variant allele and CD risk in a total of 5338 cases and 5076 controls (Pooled ORs = 1.35, 95% CI: 1.12-1.38; P = 0.0001) (Figure 1). In the other hand, no association with UC was found when evaluating disease risk in 2592 patients and 3622 controls (Table 2), OR = 1.27, 95% CI = 0.95-1.69; P = 0.20 (Figure 2). Heterogeneity in odds ratios between studies was evidenced for CD (Q = 54.5, 23 df, P = 0.0002, I^2 = 57.6%) and UC (Q = 43.4%, 14 df, P = 0.0001, I^2 = 67.8).

Combining Asp299Gly results for CD and UC (6115 cases and 3622 controls), an overall significant increased risk for IBD was observed, OR = 1.15, 95% CI = 1.03-1.30; P = 0.015 (Figure 3). However, a significant heterogeneity in allelic frequencies distribution is reported (Cochran's Q = 52.9, I^2 = 73.6%).

Based on the studies published so far combined to our results, we observed a significant association between the T allele of the TLR4Thr399Ile Polymorphism and both CD

### Table 5 Pooled analysis of studies exploring the role of TLR4 Asp299Gly in IBD

| Study                  | Sample size | Cases (Events/Total) | Controls (Events/Total) | Odds ratio | 95% CI     | P value |
|------------------------|-------------|----------------------|-------------------------|------------|------------|---------|
| Arnott et al. 2004 [31]| IBD: 480    | 85/960               | 33/378                  | 1.016      | 0.667 to 1.54 |         |
|                        | HC: 189     |                      |                         |            |            |         |
| Franchimont et al. 2004 [32]| IBD: 610    | 131/1220             | 14/278                  | 2.268      | 1.286 to 4.00 |         |
|                        | HC: 139     |                      |                         |            |            |         |
| Torok et al. 2004 [33]  | IBD: 200    | 32/400               | 12/290                  | 2.014      | 1.019 to 3.98 |         |
|                        | HC: 145     |                      |                         |            |            |         |
| Braat et al. 2005 [34]  | IBD: 637    | 92/1274              | 13/274                  | 1.563      | 0.861 to 2.83 |         |
|                        | HC: 137     |                      |                         |            |            |         |
| Gazouli et al. 2005 [36]| IBD: 205    | 25/410               | 6/200                   | 2.100      | 0.847 to 5.20 |         |
|                        | HC: 100     |                      |                         |            |            |         |
| Oostenbrug et al. 2005 [37]| IBD: 572    | 74/1144              | 27/592                  | 1.447      | 0.921 to 2.27 |         |
|                        | HC: 296     |                      |                         |            |            |         |
| Baumgart et al. 2007 [21] (1) | IBD: 262    | 14/524               | 16/404                  | 0.666      | 0.321 to 1.38 |         |
|                        | HC: 202     |                      |                         |            |            |         |
| Baumgart et al. 2007 [21] (2) | IBD: 386    | 52/772               | 49/806                  | 1.116      | 0.745 to 1.67 |         |
|                        | HC: 403     |                      |                         |            |            |         |
| Browning et al. 2007 [42]   | IBD: 796    | 101/1592             | 44/832                  | 1.213      | 0.843 to 1.74 |         |
|                        | HC: 416     |                      |                         |            |            |         |
| Riis et al. 2007 [43]   | IBD: 615    | 85/1230              | 152/1236                | 0.529      | 0.401 to 0.69 |         |
|                        | HC: 618     |                      |                         |            |            |         |
| De Ridder et al. 2007 [23] | IBD: 103    | 15/206               | 20/488                  | 1.838      | 0.921 to 3.66 |         |
|                        | HC: 103     |                      |                         |            |            |         |
| De Ridder et al. 2007 [23] | IBD: 604    | 96/1208              | 20/488                  | 2.020      | 1.233 to 3.31 |         |
|                        | HC: 244     |                      |                         |            |            |         |
| Rigoli et al. 2008 [45]  | IBD: 178    | 13/356               | 8/206                   | 0.938      | 0.382 to 2.30 |         |
|                        | HC: 103     |                      |                         |            |            |         |
| Manolakis et al. 2013 [46]  | IBD: 350    | 61/700               | 33/548                  | 1.490      | 0.960 to 2.31 |         |
|                        | HC: 274     |                      |                         |            |            |         |
| Our study 2014         | IBD:117    | 15/234               | 10/224                  | 1.466      | 0.644 to 3.33 |         |
|                        | HC:112     |                      |                         |            |            |         |
| Total (fixed effects)  | 891/12230  | 457/7244             | 1,154                  | 1,021 to 1,30 | 0.015     |
| Total (random effects) | 891/12230  | 457/7244             | 1,306                  | 1,006 to 1,69 |         |
and UC risk (Figures 4 and 5). As well, TLR4 Thr399Ile variant increased the overall IBD susceptibility when combining CD and UC results (OR = 1.46, 95%CI: 1.21-1.76; P < 0.0001) for a total of 3392 cases and 1773 controls (Figure 6).

In the present meta-analysis, we did not observed heterogeneity between studies for TLR4 Thr399Ile Polymorphism distribution in CD (Q = 9.05, DF = 11, I² = −21.54%; P = 0.62), UC (Q = 5.18, DF = 8, I² = −54.4%; P = 0.73) and IBD (Cohran’sQ = 7.84, DF = 10, I² = −27.5%; P = 0.64).

Genotype-phenotype correlation was investigated; demographic and clinical characteristics of CD and UC patients according to TLR4 polymorphisms are shown in (Tables 9 and 10). A significant association was found between the need for surgery and possession of one or more Asp299Gly variant alleles in UC patients (P = 0.004). The presence of TLR4 variant alleles was not associated with smoking habits, age of diagnosis, disease location and behavior, family history and presence of extra-intestinal manifestations. Separate analyses in men and women did not reveal sex related associations. None of our UC patients had an appendectomy. The correlation study between Thr399Ile polymorphism and CD or UC didn’t allow to associate TLR4 genotype with a particular phenotype.

**Discussion**
Given the evidence that an altered innate immune response and chronic inflammation are implicated in IBD

| Table 6 Pooled analysis of studies exploring the role of TLR4 Thr399Ile in IBD |
|-----------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Study                            | Sample size | Cases (Events/Total) | Controls (Events/Total) | Odds         | 95% CI      | P value      |
| Torok et al. 2004 [33]           | IBD: 200   | 38/400                 | 12/290                  | 2.432        | 1.24 to 4.74 |             |
|                                  | HC: 145    |                        |                        |              |             |             |
| Gazouli et al. 2005 [36]         | IBD: 205   | 4/410                  | 2/200                   | 0.975        | 0.17 to 5.37 |             |
|                                  | HC: 100    |                        |                        |              |             |             |
| Oostenbrug et al. 2005 [37]      | IBD: 721   | 93/1442                | 29/598                  | 1.353        | 0.88 to 2.07 |             |
|                                  | HC: 299    |                        |                        |              |             |             |
| Zouiten-Mekki et al. 2009 [40]   | IBD: 120   | 15/240                 | 8/160                   | 1.267        | 0.52 to 3.06 |             |
|                                  | HC: 80     |                        |                        |              |             |             |
| Browning et al. 2007 [42]        | IBD: 794   | 106/1588               | 46/832                  | 1.222        | 0.85 to 1.74 |             |
|                                  | HC: 416    |                        |                        |              |             |             |
| De Ridder et al. 2007 [23]       | IBD: 707   | 106/1414               | 22/488                  | 1.717        | 1.07 to 2.75 |             |
|                                  | HC: 244    |                        |                        |              |             |             |
| Rigoli et al. 2008 [45]          | IBD: 178   | 12/356                 | 6/206                   | 1.163        | 0.43 to 3.14 |             |
|                                  | HC: 103    |                        |                        |              |             |             |
| Manolakis et al. 2013 [46]       | IBD: 350   | 61/700                 | 33/548                  | 1.490        | 0.96 to 2.31 |             |
|                                  | HC: 274    |                        |                        |              |             |             |
| Our study 2014                   | IBD: 117   | 10/234                 | 3/224                   | 3.289        | 0.89 to 12.11 |             |
|                                  | HC: 112    |                        |                        |              |             |             |
| Total (fixed effects)            |            | 445/6784               | 161/3546                | 1.479        | 1.22 to 1.82 | 0.0001      |
| Total (random effects)           |            | 445/6784               | 161/3546                | 1.465        | 1.21 to 1.80 |             |

| Table 7 Allele and genotype frequencies of the studied polymorphisms in the group of patients with Crohn’s disease, ulcerative colitis and controls |
|-----------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Group                             | TLR4 Asp299Gly |                         | TLR4 Thr399Ile |                         |                         |                         |                         |                         |
|                                  | A | G | AA | AG | GG | C | T | CC | CT | TT |                  |
| CD (%) N = 83                    | 157 (94.6) | 9 (5.4) | 74 (89.2) | 9 (10.8) | - | 159 (95.8) | 7 (4.2) | 76 (91.6) | 7 (8.4) | - |
| UC (%) N = 34                    | 62 (91.2) | 6 (8.8) | 28 (82.4) | 6 (17.6) | - | 65 (95.6) | 3 (4.4) | 32 (94.1) | 1 (2.9) | 1 (2.9) |
| Controls (%) N = 112             | 214 (95.5) | 10 (4.5) | 103 (92.0) | 8 (7.1) | 1 (0.9) | 221 (98.7) | 3 (1.3) | 109 (97.3) | 3 (2.7) | - |
pathogenesis, genetic influence of pattern recognition receptors was clearly suggested as a trigger of CD and UC. Several efforts were undertaken to demonstrate associations of the human TLR4 gene (Gene map locus 9q32-q33) with IBD and its clinical manifestation. Attention was focused on co-segregating SNPs located in exon 3 of TLR4 causing amino acid exchanges at positions 299 (Asp299Gly) and 399 (Thr399Ile) which are located in the extracellular domain of the receptor [17,48]. Association of TLR4 Asp299Gly with CD was first reported by Braat et al. [30] subsequent studies have had divergent results and showed strong evidence of ethnic differences. In view of the discrepant data regarding the association of the TLR4 gene with IBD and its clinical complications, we investigated for the first time the potential influence of TLR4 SNPs in the susceptibility to IBD in a cohort of Moroccan patients. However, the statistical power was very low and could be considered a limitation in this study. Our study showed that the GG genotype was not found in both CD and UC patients. No significant differences were observed in allele frequencies of the TLR4 Asp299Gly among patients and controls. In addition, although slightly increased frequencies of the mutant alleles were encountered, we were not able to identify a significant difference in allele distributions of the TLR4 Thr399Ile in our case control study. In line with our results, a Tunisian study that genotyped 90 patients with CD and 80 healthy individuals for the Asp299Gly and Thr399Ile polymorphisms, reported the absence of association between CD and TLR4 gene in a north African population [40]. Although the Tunisian CD population showed a similar overall pattern of allelic frequencies, it is of some note that the genotype-phenotype correlation revealed divergent results. While the Thr399Ile variant allele was associated with early disease onset in Tunisian patients, no correlation with a particular phenotype was observed for this polymorphism in the Moroccan patients. Our study showed that the presence of Asp299Gly variant allele was associated with the need for surgery in UC patients (P = 0.004). Furthermore, the occurrence of one Asp299gly risk allele in CD patients was suggestive

| SNP        | Trait | Genotype/Allele | OR   | CI    | P Value |
|------------|-------|-----------------|------|-------|---------|
| Asp299Gly  | CD    | AG              | 1.57 | (0.58-4.25) | 0.38    |
|            |       | G               | 1.23 | (0.49-3.09) | 0.66    |
|            |       | AG              | 2.76 | (0.88-8.61) | 0.08    |
|            |       | G               | 2.07 | (0.72-5.92) | 0.17    |
| Thr399Ile  | CD    | CT              | 3.35 | (0.84-13.35) | 0.09    |
|            |       | T               | 3.24 | (0.83-12.74) | 0.09    |
|            | UC    | CT              | 1.14 | (0.11-11.29) | 0.91    |
|            |       | TT              | 10.11 | (0.40-254.1) | 0.16    |
|            |       | T               | 3.4  | (0.67-17.25) | 0.14    |

Table 8 Odds ratios and P values for association of TLR4 variants with IBD status

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Asnani et al. 2004
Franckenne et al. 2004 (1)
Franckenne et al. 2004
Torek et al. 2004
Braat et al. 2005
Brandt et al. 2005
Lakatos et al. 2005
Guern et al. 2005
Ouwenhuyse et al. 2005
Oudheusden et al. 2005
Fiers et al. 2005 (3)
Fiers et al. 2005 (2)
Zuurstra-Molik et al. 2005
Hong et al. 2007
Baumgart et al. 2007 (1)
Baumgart et al. 2007 (2)
Browning et al. 2007
De Ridder 2007
De Ridder et al. 2007
Rice et al. 2007
Hume et al. 2008
Rieger et al. 2008
Manolaki et al. 2013
Our study 2014
Total (fixed effects)
Total (random effects)
Pooled OR= 1.35 (1.12-1.63)

Figure 1 Forest plots for the association of TLR4 A299G and risk of CD.

Test for heterogeneity
Q= 54.5, DF= 23,
P= 57.6%, P= 0.0002
of a trend of association with smoking habits ($P = 0.04$) that was no more observed after correction for multiple testing.

Being in linkage disequilibrium, TLR4 mutant alleles are known to be inherited in the form of Asp299Gly/Thr399Ile haplotype [48]. In a German cohort, the co-segregation between mutant alleles represented 100% in controls, whereas it was not complete in CD and UC patients: 94% and 86% respectively [33]. These observations contrast our findings where simultaneous presence of the mutated alleles was only observed in 33% of controls, 33% of UC and 60% of CD patients.

![Figure 2 Forest plots for the association of TLR4 A299G and risk of UC.](image)

![Figure 3 Forest plots for the association of TLR4 A299G and risk of IBD.](image)
Results on the relationship of Asp299Gly SNP alone or in combination with Thr399Ile with IBD are inconsistent between studies. No difference in TLR4 allele frequency between IBD patients and controls was observed in Hungarian [35], Saudi Arabian [47], Southern Italian [45], New Zealandian [41] and EC-IBD [43] study groups. Genetic heterogeneity within Europe was evidenced by Arnott et al. when reporting lack of association of TLR4 and CD14 variants in Scottish and Irish CD patients [31]. Moreover, Baumgart DC et al. reported...
an association between IBD and the CD14 c.1-260C T promoter but not with the TLR4 (p.D299G) variant in Germans and Hungarians [21]. Interestingly, the heterozygous and homozygous pattern for the mutated allele was not detected in any of the individuals from the Japanese [49], Korean [24], Chinese Han population [25] and Zhuang population from the Guangxi Zhuang Autonomous Region of China [26]. TLR4 was linked to an increased IBD (CD or UC) risk in many other diverse investigations. Significant associations were found in patients drawn from Belgian [32], German [22,33], Greek [36,46] and Dutch [34] populations. In addition, several meta-analyses provided evidence that the Asp299Gly SNP is associated with CD and IBD in Caucasians [27,31]. Overall, there was inescapable evidence for considerable genetic heterogeneity. This observation has been explained by geographic and ethnicity-related gene effect on disease susceptibility [51]. Our results showed that the distribution of the risk alleles varies between both TLR4 polymorphisms. Therefore, we offer additional evidence for differences in the contribution of individual genetic determinants between populations.

Browning et al., argued that negative studies with results that do not achieve statistical significance can still contribute evidence for association, having important implications for the first generation of whole genome association studies [42].

In view of the role of potential confounders related to the present study and to discrepant results between populations, it is likely that the contribution of different sample size, selection bias, phenotypic heterogeneity and population stratification in case control studies cannot be ruled out. These data demonstrate further the real difficulties in candidate gene analysis in complex diseases. Moreover, given that IBD is a polygenic disease it is provided that association studies will reveal various sets of susceptible genes. Therefore, further large-scale studies are required to obtain a clear insight into the impact of the pattern recognition receptors in the

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**Figure 6** Forest plots for the association of TLR4 T399I and risk of IBD.

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pathophysiological and immunogenetic aspects of IBD and to explore the contribution of other genes involved in various processes.

**Conclusions**
In the present study, we have demonstrated that the common mutations in the *TLR4* gene are not associated with IBD in a sample of Moroccan patients. However, our dataset contributed to the significant association observed in TLR4 meta-analysis.

It is likely that the distribution of *TLR4* gene polymorphisms have ethnic differences. Our data suggests that other genetic and environmental factors may play a role in IBD susceptibility and behavior in this population. However, because of the relatively small sample size, additional well-powered studies are needed to confirm our findings.

### Table 9 Genotype-phenotype correlations in patients with Crohn’s disease

| Parameter                | N    | Asp299gly (%) | P value | Chi-square test | Thr399Ile (%) | P value | Chi-square test |
|--------------------------|------|---------------|---------|----------------|---------------|---------|----------------|
|                          |      | AA            | AG      | GG             | CC            | CT      | TT             |
| Age of onset             | 83   | 0.48          | 1.45    | 0.59           | 1.05          |
| <17 years                | 10   | 10 (100.0)    | -       | 10 (100.0)     | -             |
| 17-40                    | 63   | 55 (87.3)     | 8 (12.7) | 57 (90.5)     | 6 (9.5)       |
| >40 years                | 10   | 9 (90.0)      | 1 (10.0) | 9 (90.0)      | 1 (10.0)      |
| Sex                      | 83   | 0.35          | 0.87    | 0.17           | 1.92          |
| Woman                    | 25   | 24 (96.0)     | 1 (4.0)  | 25 (100.0)    | -             |
| Man                      | 58   | 50 (86.2)     | 8 (13.8) | 51 (87.9)     | 7 (12.1)      |
| Type                     | 83   | 0.54          | 2.14    | 0.36           | 3.23          |
| Fistulizing              | 35   | 31 (88.6)     | 4 (11.4) | 33 (94.3)     | 2 (5.7)       |
| Non fistulizing non stenosing | 24 | 20 (83.3)     | 4 (16.7) | 20 (83.3)     | (16.7)        |
| Stenosing                | 17   | 16 (94.1)     | 1 (5.9)  | 16 (94.1)     | 1 (5.9)       |
| Fistulizing stenosing    | 7    | 7 (100.0)     | -       | 7 (100.0)     | -             |
| Localization             | 83   | 0.80          | 1.67    | 0.94           | 0.82          |
| L1                       | 30   | 26 (86.7)     | 4 (13.3) | 27 (90.0)     | 3 (10.0)      |
| L2                       | 20   | 17 (85.0)     | 3 (5.0)  | 18 (90.0)     | 2 (10.0)      |
| L3                       | 26   | 24 (92.3)     | 2 (6.7)  | 24 (92.3)     | 2 (7.7)       |
| L4                       | 3    | 3 (100.0)     | -       | 3 (100.0)     | -             |
| L4 + L2                  | 4    | 4 (100.0)     | -       | 4 (100.0)     | -             |
| Smoking                  | 83   | 0.04          | 4.44    | 0.17           | 1.92          |
| Presence                 | 33   | 26 (78.8)     | 7 (21.2) | 28 (84.8)     | 5 (15.2)      |
| Absence                  | 50   | 48 (96.0)     | 2 (4.0)  | 48 (96)       | 2 (4.0)       |
| SFC                      | 83   | 0.91          | 0.01    | 0.76           | 0.09          |
| Presence                 | 4    | 4 (100.0)     | -       | 4 (100.0)     | -             |
| Absence                  | 79   | 70 (88.6)     | 9 (11.4) | 72 (91.1)     | 7 (8.9)       |
| Appendectomy             | 83   | 0.84          | 0.04    | 0.58           | 0.30          |
| Presence                 | 12   | 11 (91.7)     | 1 (8.3)  | 10 (83.3)     | 2 (16.7)      |
| Absence                  | 71   | 63 (88.7)     | 8 (11.3) | 66 (93.0)     | 5 (7.0)       |
| EIM                      | 83   | 0.97          | 0.001   | 0.97           | 0.001         |
| Presence                 | 42   | 38 (90.5)     | 4 (9.5)  | 39 (92.9)     | 3 (7.1)       |
| Absence                  | 41   | 36 (87.8)     | 5 (12.2) | 37 (95.1)     | 4 (4.9)       |
| Surgery                  | 83   | 0.50          | 0.45    | 0.45           | 0.57          |
| Presence                 | 41   | 38 (92.7)     | 3 (7.3)  | 39 (95.1)     | 2 (4.9)       |
| Absence                  | 42   | 36 (85.7)     | 6 (14.3) | 37 (88.1)     | 5 (11.9)      |

SFC: similar familial cases; EIM: extra intestinal manifestations; N: total number; AA: wild type TLR4 Asp299gly, AG: TLR4 Asp299gly heterozygous variant, GG: TLR4 Asp299gly homozygous variant; CC: wild type TLR4 Thr399Ile, CT: TLR4 Thr399Ile heterozygous variant, TT: TLR4 Thr399Ile homozygous variant.
Abbreviations
IBD: Inflammatory bowel disease; CD: Crohn’s disease; UC: Ulcerative colitis; PRR: Pattern recognition receptors.

Competing interests
The authors declare that they have no competing interests.

Authors contributions
NS out the molecular genetic studies, participated in the recruitment of patients and drafted the manuscript. BD performed the statistical analysis. NS participated in recruitment of patients and clinical data collection. YZ revised the manuscript. WB coordinated patients’ recruitment and provided the clinical data. SN conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

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Table 10 Genotype-phenotype correlations in patients with ulcerative colitis

| Parameter               | N | Asp299gly (%) | P value | Chi-square test | Thr399Ile (%) | P value | Chi-square test |
|-------------------------|---|---------------|---------|-----------------|---------------|---------|-----------------|
| Age of onset            | 34 |               | 0.35    | 0.89            | 19 (95.0)     | 1 (5.0) | 0.34            | 2.13 |
| <17 years               | -  | -             | -       | -               | -             | -       | -               | -   |
| 17-40                   | 20 | 18 (90.0)     | -       | 19 (95.0)       | 1 (5.0)       | -       | -               | -   |
| >40 years               | 14 | 10 (71.4)     | 4 (28.6) | 13 (92.9)       | -             | 1 (7.1) | -               | -   |
| Sex                     | 34 |               | 0.89    | 0.018           | 14 (93.3)     | 1 (6.7) | 0.36            | 2.06 |
| Woman                   | 15 | 13 (86.7)     | 2 (13.3) | 18 (94.7)       | -             | 1 (5.3) | -               | -   |
| Man                     | 19 | 15 (78.9)     | 4 (21.1) | 18 (94.7)       | -             | 1 (5.3) | -               | -   |
| Extent of the disease   | 34 |               | 0.31    | 3.55            | 1 (100.0)     | -       | 0.16            | 9.34 |
| E1                      | 4  | 2 (50.0)      | 2 (50.0) | 3 (75.0)        | -             | 1 (25.0) | -               | -   |
| E2                      | 15 | 13 (86.7)     | 2 (13.3) | 15 (100.0)      | -             | -       | -               | -   |
| E3                      | 2  | 2 (100.0)     | -       | 2 (100.0)       | -             | -       | -               | -   |
| E4                      | 13 | 11 (84.6)     | 2 (15.4) | 12 (92.3)       | 1 (7.7)       | -       | -               | -   |
| SFC                     | 34 |               | 0.39    | 0.74            | 1 (100.0)     | -       | 0.97            | 0.06 |
| Presence                | 1  | 1 (100.0)     | -       | 1 (100.0)       | -             | -       | -               | -   |
| Absence                 | 33 | 27 (81.8)     | 6 (18.2) | 31 (93.9)       | 1 (3.0)       | 1 (3.0) | -               | -   |
| Smoking                 | 34 |               | 0.93    | 0.008           | 8 (88.9)      | -       | 0.20            | 3.17 |
| Presence                | 9  | 7 (77.8)      | 2 (22.2) | 8 (88.9)        | -             | 1 (11.1) | -               | -   |
| Absence                 | 25 | 21 (84.0)     | 4 (16.0) | 24 (96.0)       | 1 (4.0)       | -       | -               | -   |
| EIM                     | 34 |               | 0.89    | 0.018           | 18 (94.7)     | -       | 0.35            | 2.05 |
| Presence                | 19 | 15 (78.9)     | 4 (21.1) | 18 (94.7)       | -             | 1 (5.3) | -               | -   |
| Absence                 | 15 | 13 (86.7)     | 2 (13.3) | 14 (93.3)       | 1 (6.7)       | -       | -               | -   |
| Surgery                 | 34 |               | 0.004   | 8.3             | 5 (83.3)      | 1 (16.7) | 0.08            | 4.97 |
| Presence                | 6  | 4 (66.7)      | -       | 5 (83.3)        | 1 (16.7)      | -       | -               | -   |
| Absence                 | 28 | 26 (92.9)     | 2 (7.1)  | 27 (96.4)       | 1 (3.6)       | -       | -               | -   |

SFC: similar familial cases; EIM: extra intestinal manifestations; N: total number; AA: wild type TLR4 Asp299gly; AG: TLR4 Asp299gly heterozygous variant; GG: TLR4 Asp299gly homozygous variant; CC: wild type TLR4 Thr399Ile; CT: TLR4 Thr399Ile heterozygous variant; TT: TLR4 Thr399Ile homozygous variant.

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