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

D Franchimont, S Vermeire, H El Housni, M Pierik, K Van Steen, T Gustot, E Quertinmont, M Abramowicz, A Van Gossum, J Devière, P Rutgeerts

Background and aims: Elicitation of an innate immune response to bacterial products is mediated through pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) and the NODs. The recently characterised Asp299Gly polymorphism in the lipopolysaccharide (LPS) receptor TLR4 is associated with impaired LPS signalling and increased susceptibility to Gram negative infections. We sought to determine whether this polymorphism was associated with Crohn’s disease (CD) and/or ulcerative colitis (UC).

Methods: Allele frequencies of the TLR4 Asp299Gly polymorphism and the three NOD2/CARD15 polymorphisms (Arg702Trp, Gly908Arg, and Leu1007fsinsC) were assessed in two independent cohorts of CD patients (cohort 1, n = 334; cohort 2, n = 114), in 163 UC patients, and in 140 controls. A transmission disequilibrium test (TDT) was then performed on 318 inflammatory bowel disease (IBD) trios.

Results: The allele frequency of the TLR4 Asp299Gly polymorphism was significantly higher in CD (cohort 1: 11% v 5%, odds ratio (OR) 2.31 (95% confidence interval (CI) 1.28–4.17), p = 0.004; and cohort 2: 12% v 5%, OR 2.45 (95% CI 1.28–4.17), p = 0.007) and UC patients (10% v 5%, OR 2.05 (95% CI 1.07–3.93), p = 0.027) compared with the control population. A TDT on 318 IBD trios demonstrated preferential transmission of the TLR4 Asp299Gly polymorphism from heterozygous parents to affected children (T/U: 68/34, p = 0.01). Carrying polymorphisms in both TLR4 and NOD2 was associated with a genotype relative risk (RR) of 4.7 compared with a RR of 2.6 and 2.5 for TLR4 and NOD2 variants separately.

Conclusion: We have reported on a novel association of the TLR4 Asp299Gly polymorphism with both CD and UC. This finding further supports the genetic influence of PRRs in triggering IBD.
susceptibility to Gram negative infections.\textsuperscript{21, 22} While a recent study suggested its limited impact on LPS stimulated human monocytes,\textsuperscript{23} this polymorphism, located in the extracellular LRR domain of TLR4, impairs LPS signalling and results in decreased nuclear factor \( \kappa \) B activation in transfection experiments.\textsuperscript{20} Even if a single polymorphism has a relative in vivo penetrance compared with the many other functional gene variants involved in LPS signalling, the TLR4 Asp299Gly polymorphism could produce similar phenotypic consequences to those observed with NOD2 polymorphisms and participate in the pathogenesis of CD. In this paper, we sought to determine whether this polymorphism was associated with CD and/or ulcerative colitis (UC), and examined its impact, alone or in combination with NOD2 polymorphisms, on a particular CD or UC phenotype.

\section*{PATIENTS AND METHODS}

\textbf{Human subjects and phenotypic analyses}

Two populations of CD patients seen at University Hospital Gasthuisberg, Leuven (cohort 1, \( n = 334 \)) and Erasme Hospital, Brussels (cohort 2, \( n = 114 \)) were examined for clinical characteristics, biological markers, and genotype, as well as one cohort of UC patients (\( n = 163 \), Gasthuisberg) and a control population of blood donors (\( n = 140 \), Gasthuisberg). Almost all CD patients in cohort 1 were White Caucasian (98\%, \( n = 328 \)); four patients were Ashkenazy Jewish and two Moroccan. In cohort 2, CD patients were as follows: White Caucasian (93\%, \( n = 106 \)), Moroccan (\( n = 6 \)), and Ashkenazy Jewish (\( n = 2 \)). Controls and UC patients were all White Caucasian. CD cohort 1 included related patients while cohort 2 did not. In cohort 1, 192/334 (57.5\%) patients reported having at least one first or second degree relative with IBD. In total, there were 54 affected sibling pairs, 18 parent-child affected pairs, and 24 second degree affected relatives pairs included in this cohort. UC patients were included in a single cohort because of the low incidence of UC in Belgium and Northern France and hence the low recruitment rate of UC in our centres. Subsequently, a population of 318 trios (one affected offspring and both parents) from the University Hospital Gasthuisberg, Leuven, was analysed to perform the transmission disequilibrium test (TDT). This population consisted of CD trios (\( n = 252 \)), UC trios (\( n = 61 \)), and indeterminate colitis (IC) trios (\( n = 5 \)). A total of 216 CD trios were derived from the 334 CD patients from cohort 1. The 61 UC trios were derived from the 163 UC patients. CD and UC patients were diagnosed according to clinical, radiological, endoscopic, and pathological criteria, as previously described.\textsuperscript{24} The following clinical characteristics were analysed: age at diagnosis, sex, family history, disease location, surgery, smoking, and extraintestinal manifestations (tables 1, 2). CD location and behaviour were assessed according to the Vienna classification (table 1).\textsuperscript{25} Informed written consent was obtained from all study participants. This study was conducted after review and approval of the ethics committees of Gasthuisberg, Leuven, and Erasme Hospitals, Brussels.

\textbf{Serological marker ASCA}

Anti-Saccharomyces cerevisiae antibody (ASCA) was measured by a standardised ELISA using mannan immobilised on the solid phase of a microtitre plate, according to the manufacturer’s instructions, as previously described (Medipan Diagnostica, Selchow, Germany).\textsuperscript{26} Qualitative evaluation of the results was assessed by calculating the binding index (BI): \( BI = OD \) (sample)/OD (cut off control). ASCA IgG and IgA were considered positive at a BI >1.0.

\textbf{Genotyping}

DNA was extracted from whole venous blood from all patients and stored at \(-80^\circ\text{C} \).
G67950), and Leu1007InsC (SNP13, Genbank accession No G67955). All DNA amplifications were carried out in 15 μl polymerase chain reaction (PCR) reactions. For Arg702Trp, 35 PCR cycles at an annealing temperature of 56°C were performed with primers 5' AGA TCA CAG GAG CCT TCC TG -3' (forward) and 5' CAC GCT TCT GGC CTC ACC-3' (reverse), followed by MspI digestion at 37°C overnight, resulting in visualisation of either 54+76+20+35 bp bands (wild-type) or 130+20+35 bp bands (mutated allele) on a 3% agarose gel. Gly908Arg was amplified using primers 5' CCC AGC TCC TCC CTC TTC-3' and 5' AAG TCT GTA ATG TAA AGC CAC-3' (annealing temperature 55°C, 35 cycles), followed by HhaI digestion, resulting in an intact fragment for the wild-type (380 bp) or two bands of 131 bp (fs3020insC) (annealing temperature 55°C, 30 cycles). For Leu1007InsC, primers used were forward 5' TCT GCC ATT CC-3' (annealing temperature 55°C, 35 cycles), followed by ApaI digestion at 25°C overnight, resulting in an intact band of 151 bp (wild-type allele) or two bands of 131+20 bp (fs3020insC) (annealing 55°C, 30 cycles).

**Table 3** Toll-like receptor 4 (TLR4) genotypes in the study populations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CD cohort 1 (n = 334)*</th>
<th>CD cohort 2 (n = 113)**</th>
<th>UC (n = 163)***</th>
<th>Controls (n = 139)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>265 (79.3%)</td>
<td>88 (77.9%)</td>
<td>133 (81.6%)</td>
<td>126 (90.6%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>65 (19.5%)</td>
<td>24 (21.2%)</td>
<td>28 (17.2%)</td>
<td>12 (8.6%)</td>
</tr>
<tr>
<td>Homozygous mutant</td>
<td>4 (1.2%)</td>
<td>1 (0.9%)</td>
<td>2 (1.2%)</td>
<td>1 (0.8%)</td>
</tr>
<tr>
<td>Allele frequencies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minor allele</td>
<td>10.9%</td>
<td>11.5%</td>
<td>9.8%</td>
<td>5.0%</td>
</tr>
</tbody>
</table>

CD, Crohn’s disease; UC, ulcerative colitis.
All p values are compared with controls: *p = 0.012, **p = 0.017, ***p = 0.08.

**Statistical analyses**
Allele frequencies were tested for the Hardy-Weinberg equilibrium. Cases and controls were compared using Pearson’s χ² test. Statistical analyses were performed using Genmod procedure in SAS/STAT taking into account the familial structure (SAS V8-Genmod procedure). Simple and multiple logistic regression models were used to analyse genotypetype-phenotype relationships, accounting for familial structure present in the data, with compound symmetry
correlation working assumptions whether appropriate (SAS V8-Genmod procedure). Results are expressed as odds ratios (OR) with corresponding 95% confidence intervals (95% CI). Significance level was predefined at 0.05. For calculation of the genotype relative risk, we first deduced the genotype absolute risk (GAR) of the different genotypes for the development of CD. Assuming a population prevalence of 1/1000 for CD, GAR for development of CD in a wild-type individual is $\text{GAR}_{\text{wild-type}} = \frac{p}{1000}$, with $p$ being the relative proportion of CD patients versus controls carrying the wild type (−/−) genotype. The same calculations were made for heterozygous and homozygous mutant genotypes. The genotype relative risk (GRR) was obtained by assuming that $\text{GRR}_{\text{wild-type}} = 1$ and $\text{GRR}_{\text{heterozygous}} = \frac{\text{GAR}_{\text{heterozygous}}}{\text{GAR}_{\text{wild-type}}}$. Alternatively, OR are a good estimate for GRR and give similar values when the probability of the outcome of interest is small, as in this case with CD where the population prevalence is low.26

RESULTs

Demographic and clinical characteristics of the CD (cohorts 1 and 2) and UC patients are shown in tables 1 and 2. There were more women than men in CD cohort 1, with a younger age at diagnosis and a higher prevalence of a familial history. More patients suffered from penetrating diseases in CD cohort 2 (tables 1, 2).

We first examined the prevalence of the TLR4 Asp299Gly genotype in CD and UC patients. There were significantly more CD patients in cohort 1 carrying the TLR4 Asp299Gly variant compared with the control population (20.7% v 13.5%; $p = 0.017$) (table 3). Mutant allele frequency was significantly higher in CD patients from cohorts 1 (11% v 5%, OR 2.31 (95% CI 1.28–4.17); $p = 0.004$) and 2 (12% v 5%, OR 2.45 (95% CI 1.24–4.816); $p = 0.007$) compared with the control population (combined cohorts 1 and 2: 11% v 5%, OR 2.35 (95% CI 1.32–4.18); $p = 0.001$) (fig 1A). Similarly, for UC patients, the allele frequency of the TLR4 polymorphism was significantly higher than in controls (10% v 5%, OR 2.05 (95% CI 1.072–3.930); $p = 0.027$) (fig 1A). TLR4 allele frequencies were in Hardy-Weinberg equilibrium in all cohorts and controls. In order to further assess the relevance of the association of the TLR4 Asp299Gly polymorphism with CD, TDT testing was performed on 318 IBD trios (table 5). Overtransmission of the TLR4 Asp299Gly polymorphism from heterozygous parents to affected children was observed in the CD trios, with 52 transmissions versus 29 non-transmissions ($\chi^2 = 6.53, df = 1, p = 0.01$). When the CD, UC, and IC trios were combined, this overtransmission was still observed (64 transmissions versus 38 non-transmissions; $\chi^2 = 6.63, df = 1, p = 0.01$). We then examined whether this TLR4 polymorphism could be related to particular CD or UC phenotypes. Detailed analysis did not show any association of the TLR4 polymorphism with either CD or UC patient subgroups (data not shown). Thus the TLR4 Asp299Gly polymorphism was significantly associated with CD and UC without influencing disease phenotype.

TLR4 and NOD2 are both PRRs involved in bacterial recognition.8 Three NOD2 variants (Arg702Trp, Gly908Arg, and Leu1007FsinsC) have been reported previously to be associated with CD.11 12 We then sought to explore the influence of the interaction between the TLR4 Asp299Gly polymorphism and the three NOD2 variants on the relative risk (RR) of CD and on CD phenotype. Consistent with previous studies, all three NOD2 polymorphisms were significantly associated with CD, as observed in cohort 1 (44.9% v 22.2%, OR 4.4 (95% CI 2.99–6.48); $p<0.001$) and cohort 2 (41.2% v 22.2%, OR 2.19 (95% CI 1.36–3.51); $p = 0.004$) but were not associated with UC (OR 0.76 (95% CI 0.45–1.27); $p = 0.28$) (table 7). When examining the interaction between the TLR4 Asp299Gly polymorphism and NOD2 genotype (table 6), no interaction between TLR4 and NOD2 was observed as the allele frequency of the TLR4 Asp299Gly risk allele did not differ whether NOD2 variants were present (10.9%) or absent (10.2%). The RR of carrying one variant in TLR4 or NOD2 was 2.652 and 2.528, respectively. Table 7 shows the RR of carriers heterozygous or homozygous for TLR4 or NOD2 variants.

### Table 4 NOD2 genotypes in the study populations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CD cohort 1 (n = 334)†††</th>
<th>CD cohort 2 (n = 114)†††</th>
<th>UC (n = 163)</th>
<th>Controls (n = 140)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>184 (55.1%)</td>
<td>67 (58.8%)</td>
<td>135 (82.9%)</td>
<td>109 (77.8%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>122 (36.5%)</td>
<td>41 (35.9%)</td>
<td>25 (15.3%)</td>
<td>28 (20.0%)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>28 (8.4%)</td>
<td>6 (5.3%)</td>
<td>3 (1.8%)</td>
<td>3 (2.2%)</td>
</tr>
<tr>
<td>Homozygous or</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>compound</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele frequencies (minor allele)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg702Trp</td>
<td>12.3%</td>
<td>11.4%</td>
<td>5.5%†††</td>
<td>6.8%</td>
</tr>
<tr>
<td>Gly908Arg</td>
<td>5.7%</td>
<td>3.9%†††</td>
<td>2.4%†††</td>
<td>2.1%</td>
</tr>
<tr>
<td>Leu1007FsinsC</td>
<td>8.9%</td>
<td>8.0%</td>
<td>1.5%†††</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

### Table 5 Transmission disequilibrium test shows overtransmission of the Toll-like receptor 4 (TLR4) polymorphism to CD patients

<table>
<thead>
<tr>
<th>TLR4 Asp299Gly</th>
<th>No of trios</th>
<th>Transmitted</th>
<th>Untransmitted</th>
<th>p Value (df = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD patients</td>
<td>252</td>
<td>52</td>
<td>29</td>
<td>0.01</td>
</tr>
<tr>
<td>IBD patients</td>
<td>318</td>
<td>64</td>
<td>38</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Inflammatory bowel disease (IBD) trios include Crohn’s disease (CD) trios (n = 252), ulcerative colitis (UC) trios (n = 61), and indeterminate colitis (IC) trios (n = 5).

### Table 6 Stratification of Toll-like receptor 4 (TLR4) allele frequency by NOD2 genotype

<table>
<thead>
<tr>
<th>NOD2 genotype in CD</th>
<th>TLR4 Asp299Gly allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>10.9%</td>
</tr>
<tr>
<td>Heterozygous, homozygous, and compound heterozygous combined</td>
<td>10.2%</td>
</tr>
</tbody>
</table>

CD, Crohn’s disease.
Table 7  Relative risks (expressed as odds ratios) for the Toll-like receptor 4 (TLR4) and NOD2 genotypes

<table>
<thead>
<tr>
<th>NOD2 mutations</th>
<th>Wild-type</th>
<th>Heterozygous</th>
<th>Homozygous mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>251</td>
<td>163</td>
<td>34</td>
</tr>
<tr>
<td>Controls</td>
<td>109</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1</td>
<td>2.528 (1.562–4.11)</td>
<td>4.199 (1.41–20.513)</td>
</tr>
<tr>
<td>TLR4 Asp299Gly</td>
<td>353</td>
<td>89</td>
<td>5</td>
</tr>
<tr>
<td>CD</td>
<td>126</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1</td>
<td>2.652 (1.356–5.274)</td>
<td>1.780 (0.201–40.619)</td>
</tr>
</tbody>
</table>

CD, Crohn’s disease; OR, odds ratio; 95% CI, 95% confidence interval.
For TLR4 Asp299Gly, one CD patient and one control could not be genotyped.

homzygous for both TLR4 and NOD2 variants could not be measured as there were no patients with this genotype combination. The RR of each of the NOD2 polymorphisms stratified by TLR4 genotype could not be calculated because of the limited sample size. Defining the RR of CD when carrying no variants in TLR4 or NOD2 as 1, the RR in carriers of either TLR4 or NOD2 variant was 3.071, and the RR in carriers of both TLR4 and NOD2 variants was 4.73. Thus the TLR4 Asp299Gly polymorphism increases the RR of developing CD either alone or when combined with the NOD2 polymorphisms.

Phenotypic analysis demonstrated an association of the NOD2 variants with small bowel involvement and fibrostenotic disease, and a negative association with colonic involvement.16 In our CD population (cohorts 1 and 2), multiple logistic regression showed a positive and negative association with ASCA (OR 1.94 (95% CI 1.29–2.90); p = 0.001) and colonic involvement (OR 0.45 (95% CI 0.24–0.84); p = 0.012), respectively. We then examined whether CD clinical characteristics were associated with any of the four genotypes (TLR4−/NOD2+, TLR4−/NOD2−, TLR4+/NOD2+, and TLR4+/NOD2−). The NOD2+/TLR4− genotype was the only combination with a significant genotype-phenotype association. Indeed, the NOD2+/TLR4− genotype was significantly associated with ASCA (p = 0.003; OR 1.92 (95% CI 1.242–2.953)) and negatively associated with colitis (p = 0.015; OR 0.43 (95% CI 0.211–0.848)).

Analysing SNP interactions influencing the level of expression and/or function of signalling molecules will allow an understanding of the phenotypic variation in LPS transduction. LPS may first interact with soluble or membrane bound CD14 before binding to TLR4.4 The recently described C−260T promoter polymorphism is associated with increased expression and levels of soluble CD14, which may further amplify LPS signalling.21 While reported to be associated with CD or UC,23 both the genotype and allele frequencies of this polymorphism were not higher in our CD (cohort 1 and 2) and UC patients compared with controls (fig 1B). Furthermore, this polymorphism, either alone or in combination with the Asp299Gly TLR4 polymorphism, was not associated with any CD or UC clinical characteristics.

DISCUSSION

IBD results from an abnormal innate mucosal immune response to gut microflora in a genetically susceptible host.12 In this study, we report on a novel genetic association between a polymorphism in the LPS receptor TLR4, Asp299Gly, and both CD and UC. Evaluating the association of the TLR4 Asp299Gly polymorphism with predefined clinical characteristics and biological markers failed to show any impact on a particular CD or UC phenotype. The genetic association of CD with NOD2 polymorphisms and TLR4 Asp299Gly clearly suggests disturbed interactions with Gram negative and Gram positive bacteria in the triggering of CD.

Also, the association of this TLR4 variant with UC further supports the role of Gram negative bacteria in the pathogenesis of UC.24 Thus the novel association with the TLR4 polymorphism strongly suggests the genetic influence of PRRs in susceptibility to IBD.

The intestinal mucosa is continuously exposed to massive amounts of commensal and pathogenic bacteria but will hardly ever initiate a mucosal immune response to bacterial products such as LPS. The IEC is a major player in the regulation of intestinal immune response to luminal antigens. Low level TLR4 expression in IECs is believed to account for normal mucosal hyporesponsiveness to LPS.25 In line with these observations, it would be expected that the TLR4 Asp299Gly polymorphism associated with a deficient LPS response should prevent mucosal immune response to Gram negative bacteria. However, TLR4 is induced by proinflammatory cytokines and is highly expressed in IECs, resident macrophages, and dendritic cells in inflamed mucosa of IBD patients.14 15 Dendritic cell maturation and the development of adaptive immunity seem to require correct TLR4 signalling. Disruption of TLR4 signalling could engender an inappropriate innate and adaptive immune response necessary to eradicate pathogens which would result in a more severe inflammation.26 This is well illustrated in C57BL/10ScCr mice which bear loss of function mutation of TLR4 and are highly susceptible to dextran sodium sulphate induced colitis.27 Further evidence comes from generation of the derived strain, C57BL/10ScCr+ mice, which develop spontaneous colitis and demonstrate highly reactive B and T cells to conventional antigens of their enteric bacterial flora.16–18 In humans, although the functional relevance of the TLR4 Asp299Gly polymorphism has been carefully examined in vivo and in transfection experiments in vitro,20 a recent study has questioned the influence of this polymorphism on LPS signalling in human monocytes isolated from wild-type and heterozygous healthy carriers.29 In truth, these results were highly expected as only in vitro transfection experiments can reveal the functional significance of a single polymorphism. While the TLR4 Asp299Gly polymorphism might have relative in vivo penetrance given that many transducing molecules involved in the LPS signalling pathway must also bear functional polymorphisms and influence the in vivo LPS response,20 this polymorphism could predispose individuals to an altered innate immune response and chronic inflammation.

The TLR4 gene was not found to lie within a chromosomal region linked to CD in genome wide linkage studies. This is however not surprising as linkage methodologies in complex diseases can miss significant loci, and it is anticipated that biologically relevant disease associations may be established for IBD in chromosomal regions that are not previously implicated through linkage studies.15 Moreover, as CD is a heterogeneous polygenic disease, it is expected that association studies will reveal various sets of susceptible genes.
depending on the ethnic background of the study populations. It is remarkable that the NOD2 mutations associated with CD in the USA and Western Europe are indeed not detected in Spain and Scotland while positive associations were observed in three independent cohorts in Belgium and the Netherlands. Further genetic studies should resolve the impact of the PRR in the susceptibility of CD, depending on ethnic background.

In conclusion, we identified an association of the TLR4 Asp299Gly polymorphism in two independent cohorts of CD and in UC. Our findings support the importance of PRRs in the genetic control of LPS signalling in IBD. Studying SNPs in molecules involved in bacterial recognition will be essential to understand individual responses to bacterial components and define genetic backgrounds at risk of IBD.

ACKNOWLEDGEMENTS

Supported by the Belgian FNRS (to DF) and the FWO (to SV).

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Gut 2004 53: 987-992
doi: 10.1136/gut.2003.030205

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