## Immunogenetics of infection and inflammation of the urogenital and gastrointestinal tracts and probiotics

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# Chapter 4

## *TLR2*, *TLR4*, and *TLR9* haplotypes in the susceptibility to and severity of *Chlamydia trachomatis* infections in Dutch women

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## Summary

*Chlamydia trachomatis* (Ct) infections have remarkable interindividual differences in the clinical course which are for 40% based on host genetic variation including genes encoding immunoregulatory and bacteria sensing proteins. As patient-related immunomodulating factors, toll-like receptors (TLRs) are closely involved in pathogen recognition and host defence in Ct infections.

Here we study the single nucleotide polymorphisms (SNPs) and their haplotypes in *TLR2, TLR4,* and *TLR9* genes (*TLR2* +2477 G>A; *TLR2* -16934 T>A; *TLR4* +896 A>G; *TLR9* -1237 T>C and *TLR9* +2848 G>A) in relation to the susceptibility to, and severity of Ct infections. We hypothesise that infection is a complex entity of multifactorial traits in which multiple genes are involved. Therefore, we performed multigen analyses. We analyzed the 5 SNPs in a cohort of 770 Dutch Caucasian women with sexually transmitted diseases (n: 731) or tubal pathology (n: 39). We identified a trend showing a protective effect for *TLR9* -1237 CC in Ct-positive patients (p: 0.056, OR: 0.14, 95%CI: 0.02 – 1.12). Haplotype analyses show a trend for *TLR2* haplotype I (-16934 T/ +2477 G) in the protection against the development of symptoms and tubal pathology ($P_{trend}$: 0.03) after *Chlamydia* infection. In the susceptibility cohort, *TLR9* haplotype III (-1237 C / +2848 A) shows a decreasing significant trend in the development of symptoms after CT infection (p: 0.02, OR: 0.55, 95%CI: 0.33-0.91). Logistic regression of the *TLR2* haplotypes, *TLR4* +896 A>G, and *TLR9* haplotypes shows that the *TLR2* haplotype combinations AG-TA and AG-TG confer risk (OR 3.4 (p: 0,01) and OR 1.6 (p: 0,03), while the *TLR9* haplotype combination TG-TA protects against CT infections (OR: 0.4, p: 0.004)). Our study shows that both *TLR2* and *TLR9* genes and combinations do influence the clinical course of *Chlamydia* infections.

## Author summary

*Chlamydia trachomatis* (Ct) infections are the most prevalent bacterial STD know remarkable interindividual differences in the clinical course, which are for 40% based on host genetic variation including in genes encoding immune regulatory and bacteria sensing proteins. As patient-related immunomodulating factors, toll like receptors (TLRs) are closely involved in pathogen recognition and host defence in Ct infections. Here we study genetic variations of the *TLR2, TLR4,* and *TLR9* genes in relation to the susceptibility to, and severity of Ct infections. We also hypothesise that infection is a complex entity of multifactorial traits in which multiple genes are involved.

We analyzed women with or without *Chlamydia* infection, and women with or without tubal pathology. Our results show that genetic variation in *TLR9* protects against *Chlamydia* infections and symptoms of infection. Specific *TLR2* variation protects against the development of a more severe clinical course of infection while other *TLR2* variations increase the susceptibility to infection (P: 0.03 - P: 0.004). Our results provide new insight into the biological mechanisms underlying *Chlamydia* infections. These results may in the future help to identify patients at risk for a more severe clinical course of infection and result in a more tailor-made therapy.




## Introduction

The most important study in the field of host genetics for the highest prevalent bacterial cause of sexually transmitted infections with a major global health treat, *Chlamydia trachomatis* (Ct), showed recently, based on twin studies, that up to 40% of the responses to Ct infection are based on host genetics.[1] These findings shed new light on the clear differences in the clinical course of Ct infections, for the susceptibility to, and severity of (*e.g.* the development of late complications including infertility and tubal pathology) infection. They forward the field to elucidate genes involved in the observed differences in the course of infection.

Infections often remain asymptomatic and provide a huge reservoir for transmission of the infection but identification of these cases by selective screening is difficult and associated costs have to be taken into account when screening programmes are considered.[2,3] Furthermore, screening is also hampered by the emergence of new variants.[4-6] Repeated infections seem associated with severe upper genital tract pathology including pelvic inflammatory disease (PID), ectopic pregnancy, and tubal infertility in decreasing order of occurrence.[7] Due to asymptomatic and subclinical Ct infection leading to unrecognized PID, many patients may stay untreated. This causes a deteriorated reproductive health of the society and entails significant expenditures for the treatment of this pathology. Ct infections and other factors

contribute to the 10-15% subfertility observed in individuals.[8] Although other factors may cause fallopian tube damage, antibodies against *C. trachomatis* are consistently found in the serum of subfertile women with tuboperitoneal disorders.[8]

The clinical course of Ct infection shows remarkable inter-individual differences in transmission, (a)symptomatic course, persistence or clearance of infection, transmission dynamics and development of late complications. In general, the differences in clinical course could be explained by the interaction between host factors, virulence factors of the pathogen, and environmental factors such as co-infections. *Chlamydia* consists of different serovars (9, 10). No specific serovars have been identified that clearly link to the course of infection. However, more recently, the worldwide most prevalent serovars of the B serogroup were found to induce higher serologic responses than those of serogroups C and I.[11] The influence of bacterial factors on the course of infection is clear as was shown by the differences in duration of infection between mice infected with serovars D, H, and MoPn.[12,13]

More recently, CT135 has been described as a novel virulence factor for Ct infections, which might also be useful for postinfection sequelae.[14] However, since serovars and other bacterial factors do not fully explain the observed differences in clinical course, it is promising to study host immunogenetic variation in relation to *Chlamydia* infections.



Toll-like receptors (TLRs) form a major group of pattern recognition receptors (PPRs) which play an important role in the innate immune response to infections, serve as a link between innate and adaptive immunity, and are closely involved in pathogen recognition and host defence in infections including *Chlamydia*. *Chlamydiae* express a variety of ligands that could serve as potential TLR ligands, leading to the production of cytokines and in the formation of inflammasomes.[15]

TLR2 was previously described as PRR for the Ct component peptidoglycan (16). In knockout mice studies, TLR2 plays an important role as mediator in the innate immune response to Ct infection as well as in the early production of inflammatory mediators and in the development of chronic inflammatory pathology.[17] Functional SNPs in the *TLR2* gene have been described in relation to infection and inflammation.[18,19]

TLR4 has been described as PPR for *Chlamydial* lipopolysaccharide (LPS) via a receptor complex of CD14 and the accessory MD-2.[20]

Under certain circumstances, TLR4 signaling may be activated in the absence of CD14 and MD-2. Activation of TLR4 recruits myeloid differentiation protein 88 (MyD88), an intracytoplasmic downstream adaptor molecule, which activates inflammation, proliferation and apoptosis.

TLR9 is required for effective innate immune responses against bacterial pathogens. It is the receptor for bacterial DNA containing sequences of non-methylated CpG dinucleotides, which is localized and acts in the cytoplasm. CpG DNA induces immunostimulation by inducing dendritic cell maturation, a strong T-helper-1-like inflammatory response, B-cell maturation, and production of several cytokines.[21-26] The *TLR9* gene and its polymorphisms are associated with several (infectious) diseases such as *Aspergillus fumigatus* infections[27], leishmaniasis[28], HIV[29], malaria (30, 31), pneumococcal infections[32], pouchitis[33], asthma[34], and renal fibrosis.[35]

TLR2, TLR4, and TLR9 employ similar proinflammatory pathways and are an example of the redundancy in pathogen recognition mechanisms. Therefore we hypothesize that combined carriage of SNPs in these genes has a more severe impact on the immune system.

We have previously shown in murine models that SNPs in the *TLR4* and *TLR9* genes increase the risk of tubal pathology following Ct infections.[13,36] Furthermore, women with tubal pathology due to Ct infection are more often carriers of the mutant *TLR4* +896G allele compared to those without tubal pathology.[37] Knockout mice deficient for *TLR9,* appear to be protected against reinfection with Ct.[38] Wildtype mice seem to be more protected against Ct reinfection compared to the *TLR4*-deficient mice.[36] In a previous study we found an association between *TLR2* haplotype I and protection against development of tubal pathology.[39] These results indicate the importance of these genes in Ct infections and warrant further study.

We extended our previously studied cohorts of STD and subfertile subjects with and without Ct to analyse the combined impact of variation in these genes and to strengthen our previously found associations with *TLR2* +2477 G>A (Arg753Gln; rs5743708), *TLR2* -16934 T>A (rs4696480), *TLR4* +896 A>G (Asp299Gly; rs4986790), *TLR9* -1237 T>C (rs5743836), and *TLR9* +2848 G>A (Pro545Pro; rs352140). We aim to assess the role of combined genetic variation in these genes in relation to the susceptibility to (Ct+ *vs.* Ct-) and the severity (symptomatic *vs.* asymptomatic; tubal pathology (TP) *vs.* no TP) of *Chlamydia trachomatis* infections.

## Materials and methods

### *Patient populations*

*STD cohort*

Women of Dutch Caucasian (DC) origin (n = 731), under the age of 33 (range 14-33 years; median 22 years) visiting the STD outpatient clinic of the Municipal Health Service in Amsterdam were included, as described previously (40). Ct positive cases (n = 322) were defined as IgG antibody positive (Medac Diagnostika GmbH, Hamburg, Germany) and Ct DNA positive (Roche Diagnostics, Basel, Switzerland). Those without Ct infection with negative Ct-DNA test and negative Ct serology, served as controls (n = 409). Of the Ct+ women, 130 women had symptoms whereas 192 were asymptomatic.



Participants were asked to sign an informed consent and fill out a questionnaire, regarding their current complaints, varying from increased discharge, having bloody discharge during coitus or thereafter, recent abdominal pain (not gastrointestinal or menses related) and/or dysuria. A cervical swab was taken for the detection of Ct-DNA by PCR.[41] Peripheral venous blood was collected for the analysis of IgG antibodies against Ct (Medac Diagnostika GmbH, Hamburg, Germany). A titre of ≥ 1:50 was considered positive. Samples with grey zone values, *i.e.* cut off ± 10%, were repeated and considered positive when the result was positive or again within the grey zone. Infections with *Candida albicans, Neisseria gonorrhoea, Trichomonas vaginalis,* and *Herpes simplex virus 1/2* may result in symptoms similar to Ct infection. Therefore, an infection status was recorded for these microorganisms. Microorganism detection was done according to methods described previously.[40]

*Subfertility cohort*

This cohort consisted of DC women (n = 39) selected from a large cohort (n=259) visiting the department of Obstetrics and Gynaecology of the Academic Hospital Maastricht, The Netherlands, in the period between December 1990 and November 2000 because of subfertility.[42] Out of the 39 women 26 were diagnosed with clinically well-defined tubal pathology (TP) based on stringent criteria in order to compare development of tubal pathology in *C. trachomatis* IgG positive women in relation to host genetic factors.

A laparoscopy with tubal testing had been performed in these women as part of their fertility work-up. Preoperative blood was drawn from all patients for *Chlamydia* IgG antibody testing (CAT), and spare sera were cryopreserved. Two independent investigators, who were unaware of the CAT results, scored the laparoscopy reports to assess the grade of tubal pathology. Tubal pathology was defined as extensive peri-adnexal adhesions and/or distal occlusion of at least one tube at laparoscopy.[43] Subfertile women who had no peri-adnexal adhesions and had patent tubes at laparoscopy served as negative controls.

IgG antibodies to Ct were detected with a species-specific MIF test (AniLabSystems, Finland), as described previously by Land *et al.*[42], with comparable sensitivity and specificity as compared to the IgG ELISA from Medac used for the STD cohort.[44] A positive Ct IgG MIF test was defined as a titre ≥1:32.

***Immunogenetic analyses***

*DNA Extraction*

*STD cohort*

Eukaryotic DNA from PBMC was isolated using the isopropanol isolation method. In short: 100 µl PBMC in PBS were added to 600 µl L6 (Nuclisens Lysisbuffer, Organon Teknika, Boxtel, The Netherlands) and 1 µl glycogen (Roche Molecular Diagnostics, Almere, The Netherlands). The samples were incubated for 30 minutes at 65°C and left to cool at RT. An equal volume of cold (-20°C) isopropanol was added to the samples. The samples were then centrifuged (20 min at 20.000 g). The supernatant was discarded and the pellets were washed twice in 75% EtOH. The pellets were dissolved in T10 overnight at 4°C and then stored at -20°C until further analysis.



*Subfertility cohort*

Genomic DNA was extracted out of the 200 microliter cryopreserved sera using the High Pure PCR Template Preparation Kit (HPPTP kit; Roche Molecular Biochemicals, Mannheim, Germany).

***Gene polymorphisms***

Genotyping was performed using the TaqMan method (Applied Biosystems). We genotyped the following SNPs: *TLR2* +2477 G>A, Arg753Gln (rs5743708) and *TLR2* -16934 T>A (rs4696480); *TLR4* +896 A>G, Asp299Gly (rs4986790); *TLR9* -1237 T>C (rs5743836) and *TLR9* +2848 G>A, Pro545Pro (rs352140). The two TLR9 SNPs are used to tag the four haplotypes as described by Lazarus *et al.*.[34] *TLR2* -16934 has been described as a tagging SNP and divides the 11 SNP haplotype in two clades.[19] *TLR2* +2477 is used to further differentiate the haplotypes.

***Statistical analyses***

Observed genotypes were tested for deviations from the Hardy-Weinberg equilibrium. Fisher exact or $\chi^2$ tests were used to test for differences in *TLR2, TLR4,* and *TLR9* haplotypes/genotypes/carrier frequencies between the (sub) groups. P-values <0.05 were considered statistically significant. *TLR2* and *TLR9* haplotypes were inferred using PHASE v2.1.1

and SNPHAP.[45-47] We modelled combined carriage of SNPs using backward stepwise logistic regression on the *TLR2* haplotypes, *TLR4*+896 A>G, and *TLR9* haplotypes with Ct susceptibility (Ct+ *vs.* Ct-) as the outcome variable. Analyses were performed with SPSS 15 (SPSS Inc., Chicago, Illinois).

## Results

### *Susceptibility to Ct infection*

The overall genotype distributions of the *TLR2* -16934 T>A, *TLR2* +2477 G>A, *TLR4* +896 A>G, *TLR9* -1237 T>C, and *TLR9* +2848 G>A SNPs are shown in table 1a, 1b, and 1c, respectively. *TLR2* and *TLR9* haplotype distributions are shown in tables 2a and 2b, respectively. On the genotype, mutant allele carriage, and the haplotype level, the analyzed SNPs showed no statistical significance for susceptibility to Ct infections.

Subsequently we analyzed the effect of host genetics on the development of symptoms.

Ct positive patients carrying *TLR9* -1237 CC are more frequently asymptomatic (5.2%) compared to symptomatic (0.8%) (p: 0.056, OR: 0.14, 95%CI: 0.02 – 1.12).

Trend analyses in Ct positive patients (asymptomatic > symptomatic > tubal pathology) show that *TLR2* haplotype 1 (-16934 T / +2477 G) protects against a more severe clinical course of infection ($P_{trend}$: 0.03, $\chi^2_{trend}$ : 4.6) (table 2a and figure 1). *TLR9* haplotype 3 (-1237C / +2848A) is less frequent in CT+ symptomatic patients compared to CT+ asymptomatic patients (p: 0.02; OR: 0.55, 95%CI: 0.33-0.91).

Single gene analyses:

| | | | ***TLR2*** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **-16934 T>A** | | | | | | | | **+2477 G>A** | | | |
| | | **n** | **TT** | **%** | **TA** | **%** | **AA** | **%** | **GG** | **%** | **GA** | **%** | **AA** | **%** |
| **STD** | | | | | | | | | | | | | | |
| CT+ | | 322 | 91 | 28,3 | 145 | 45,0 | 86 | 26,7 | 296 | 91,9 | 22 | 6,8 | 4 | 1,2 |
| | Symp. | 130 | 33 | 25,4 | 60 | 46,2 | 37 | 28,5 | 116 | 89,2 | 13 | 10,0 | 1 | 0,8 |
| | Asymp | 192 | 58 | 30,2 | 85 | 44,3 | 49 | 25,5 | 180 | 93,8 | 9 | 4,7 | 3 | 1,6 |
| CT- | | 409 | 112 | 27,4 | 195 | 47,7 | 102 | 24,9 | 366 | 89,5 | 39 | 9,5 | 4 | 1,0 |
| **Subfertility** | | | | | | | | | | | | | | |
| CT+ | TP+ | 26 | 7 | 26,9 | 9 | 34,6 | 10 | 38,5 | 21 | 80,8 | 5 | 19,2 | 0 | 0,0 |
| | TP- | 13 | 6 | 46,2 | 5 | 38,5 | 2 | 15,4 | 13 | 100,0 | 0 | 0,0 | 0 | 0,0 |

**Table 1a:** TLR2 *SNP distribution in the STD and subfertility cohorts. CT:* C. trachomatis*; Symp: symptomatic; Asymp: Asymptomatic; TP: Tubal pathology*

| | | | | | ***TLR4*** **+896 A>G** | | | |
|---|---|---|---|---|---|---|---|---|
| | | **n** | **AA** | **%** | **AG** | **%** | **GG** | **%** |
| **STD** | | | | | | | | |
| CT+ | | 322 | 282 | 87,6 | 40 | 12,4 | 0 | 0,0 |
| | Symp. | 130 | 114 | 87,7 | 16 | 12,3 | 0 | 0,0 |
| | Asymp | 192 | 168 | 87,5 | 24 | 12,5 | 0 | 0,0 |
| CT- | | 409 | 352 | 86,1 | 57 | 13,9 | 0 | 0,0 |
| **Subfertility** | | | | | | | | |
| CT+ | TP+ | 26 | 21 | 80,8 | 5 | 19,2 | 0 | 0,0 |
| | TP- | 13 | 12 | 92,3 | 1 | 7,7 | 0 | 0,0 |

**Table 1b:** TLR4 *SNP distribution in the STD and subfertility cohorts. CT:* C. trachomatis*; Symp: symptomatic; Asymp: Asymptomatic; TP: Tubal pathology*

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | ***TLR9*** | | | | | | | |
| | | | | | **-1237 T>C** | | | | | | **+2848 G>A** | | | |
| | | **n** | **TT** | **%** | **TC** | **%** | **CC** | **%** | **GG** | **%** | **GA** | **%** | **AA** | **%** |
| **STD** | | | | | | | | | | | | | | |
| CT+ | | 322 | 239 | 74,2 | 72 | 22,4 | 11 | 3,4 | 76 | 23,6 | 152 | 47,2 | 94 | 29,2 |
| | Symp. | 130 | 102 | 78,5 | 27 | 20,8 | 1 | 0,8 | 31 | 23,9 | 61 | 46,9 | 38 | 29,2 |
| | Asymp | 192 | 137 | 71,4 | 45 | 23,4 | 10 | 5,2 | 45 | 23,4 | 91 | 47,4 | 56 | 29,2 |
| CT- | | 409 | 297 | 72,6 | 102 | 24,9 | 10 | 2,4 | 94 | 23,0 | 200 | 48,9 | 115 | 28,1 |
| **Subfertility** | | | | | | | | | | | | | | |
| CT+ | TP+ | 26 | 16 | 61,5 | 9 | 34,6 | 1 | 3,9 | 3 | 11,5 | 10 | 38,5 | 13 | 50,0 |
| | TP- | 13 | 10 | 76,9 | 3 | 23,1 | 0 | 0 | 3 | 23,1 | 6 | 46,2 | 4 | 30,8 |

**Table 1c:** TLR9 *SNP distribution in the STD and subfertility cohorts. CT:* C. trachomatis*; Symp: symptomatic; Asymp: Asymptomatic; TP: Tubal pathology*

**Haplotype analyses:**

| | | | | | ***TLR2*** **haplotypes** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **I** | | **II** | | **III** | | **IV** | |
| | | **2n** | **TG** | **%** | **AG** | **%** | **TA** | **%** | **AA** | **%** |
| **STD** | | | | | | | | | | |
| CT+ | | 644 | 299 | 46,4 | 315 | 48,9 | 28 | 4,4 | 2 | 0,3 |
| | Symp. | 260 | 112 | 43,1 | 133 | 51,2 | 14 | 5,4 | 1 | 0,4 |
| | Asymp | 384 | 187 | 48,7 | 182 | 47,4 | 14 | 3,7 | 1 | 0,3 |
| CT- | | 818 | 376 | 46,0 | 395 | 48,3 | 43 | 5,3 | 4 | 0,5 |
| **Subfertility** | | | | | | | | | | |
| CT+ | TP+ | 52 | 18 | 34,6 | 29 | 55,8 | 5 | 9,6 | 0 | 0,0 |
| | TP- | 26 | 17 | 65,4 | 9 | 34,6 | 0 | 0,0 | 0 | 0,0 |

**Table 2a:** TLR2 *haplotype distribution in the STD and subfertility cohorts. CT:* C. trachomatis*; Symp: symptomatic; Asymp: Asymptomatic;TP: Tubal pathology*

| | | | *TLR9* haplotypes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | I | | II | | III | | IV | |
| | | 2n | TG | % | TA | % | CA | % | CG | % |
| **STD** | | | | | | | | | | |
| CT+ | | 644 | 293 | 45,5 | 256 | 39,8 | 84 | 13,0 | 11 | 1,7 |
| | Symp. | 260 | 118 | 45,4 | 113 | 43,5 | 24 | 9,2 | 5 | 1,9 |
| | Asymp | 384 | 175 | 45,6 | 143 | 37,2 | 60 | 15,6 | 6 | 1,6 |
| CT- | | 818 | 376 | 46,0 | 321 | 39,2 | 109 | 13,3 | 12 | 1,5 |
| **Subfertility** | | | | | | | | | | |
| CT+ | TP+ | 52 | 16 | 30,8 | 25 | 48,1 | 11 | 21,2 | 0 | 0,0 |
| | TP- | 26 | 12 | 46,2 | 11 | 42,3 | 3 | 11,5 | 0 | 0,0 |

**Table 2b:** TLR9 *haplotype distribution in the STD and subfertility cohorts. CT:* C. trachomatis*; Symp: symptomatic; Asymp: Asymptomatic;TP: Tubal pathology*

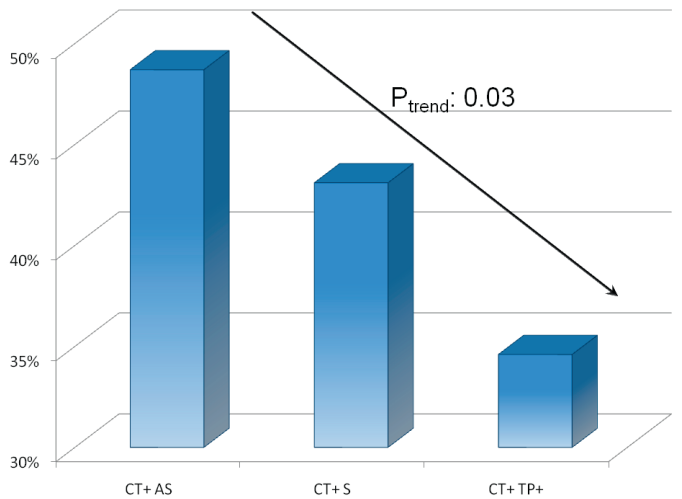


**Figure 1:** TLR2 *haplotype I shows a decreasing trend in CT+ asymptomatic, CT+ symptomatic, and CT+ tubal pathology patients*.

### *Severity of sequelae of Ct infection*

The analysed SNPs showed no statistically significant differences among the different groups in the genotype distributions.

Logistic regression of the *TLR2* haplotypes, *TLR4* +896 A>G, and *TLR9* haplotypes shows that the *TLR2* haplotype combinations AG-TA and AG-TG, and the *TLR9* haplotype combination TG-TA affect the susceptibility to CT infections. The *TLR2* haplotype combinations increase the risk of CT infections (II – III (AG-TA): p: 0,01, OR: 3,4 and II – I (AG-TG): p: 0,03, OR:

1,6, respectively), while *TLR9* haplotype combination I – II (TG-TA) decreases susceptibility to CT infections (p: 0,004, OR: 0,4). TLR4 was automatically removed from the model during the backward stepwise logistic regression, due to the lack of a significant effect in the model.

## Discussion

Immunogenetic studies have already provided insight in the pathogenesis to many infectious diseases. This study shows convincing evidence that TLRs are closely involved in pathogen recognition and host defence in Ct infections and that host genetic factors contribute to the pathogenesis as well as to the course and severity of this infection.

In this study a significant trend was observed for *TLR2* haplotype 1 (-16934 T / +2477 G) in the protection against development of symptoms and subsequent development of tubal pathology. These results corroborate our previous findings in a much smaller study where we have shown that *TLR2* haplotype 1 protects against development of tubal pathology.[39]

We did not observe significant differences in the *TLR4* genotype distributions between the cases and controls in this study in both STD and TP cohorts; although in a previous study we found a trend for the development of tubal pathology in CT DNA positive and CT IgG positive women[36] as compared to CT DNA negative and IgG negative women. Murine studies do show a role for TLR4 in *Chlamydial* infections, although human studies (including this study) indicate that the role of TLR4 in human *Chlamydia* infections might be modest.[36]

Ct positive patients carrying *TLR9* -1237 CC are less frequently symptomatic (p: 0.056, OR: 0.14, 95%CI: 0.02 – 1.12). With regard to the severity of the disease, the analysed SNPs showed no statistical significant differences among the different groups in the genotype. In the subfertility cohort, *TLR9* haplotype 3 (-1237 C / +2848 A) shows a decreasing trend in the development of symptoms (p: 0.020, OR: 0.55, 95%CI: 0.33-0.91). We have previously shown a decreasing trend in the carriage of haplotype 4 (-1237 C / +2848 G) in CT positive women without symptoms, with symptoms, and with tubal pathology.[38] Both haplotypes contain the *TLR9* -1237 C allele, suggesting a role for this allele in the protection against *Chlamydia* infections.

Logistic regression modelling of the effect of *TLR4*, *TLR2* haplotypes, and *TLR9* haplotypes showed significant effects of specific combinations of *TLR2* and *TLR9* haplotypes.

*TLR2* haplotype combinations -16934A/+2477G – -16934T/+2477A and -16934A/+2477G – -16934T/+2477G show an increased *Chlamydia* susceptibility. In both haplotype combinations the samples are heterozygous for the -16934 SNP (TA), and either heterozygous (GA) or homozygous (GG) for the +2477 SNP. The haplotype combination containing the +2477 heterozygote (-16934A/+2477G – -16934T/+2477A) has a higher risk (OR: 3.4) compared to the other haplotype combination (OR: 1.6). Haplotype analyses have shown that haplotype 1 (-16934 T / +2477 G) protects against development of symptoms and tubal pathology. The

-16934 T allele and the +2477 G allele are present in the most protective combinations, while the -16934 A allele and the +2477A allele are present in the risk combinations, suggesting that the *TLR2* alleles have an effect on the immune system.
Our results are partially corraboreted by different previous studies describing the importance of TLR2 in the recognition of *C. pneumoniae*, LPS-signalling, and the development of murine tubal pathology.[17,36,48,49] *TLR2* deficient mice were found to have significantly lower levels of inflammatory mediators in genital tract secretions during the first week of infection, and there was a significant reduction in oviduct and mesosalpinx pathology at late time points.[17] This suggested that TLR2 was the predominant receptor involved in the detection of and inflammatory response to Ct in the genital tract. Furthermore, TLR2 was described to be tightly associated with the bacteria during the intracellular phase, and that it was recruited to the inclusion membrane together with MyD88. Hereby, intracellular TLR2 was suggested to be responsible for the initiation of signal transduction events during infection with Ct.[50]
The study of Lorenz *et al*. shows that the +2477 mutant is less responsive to lipoproteins[18], and the studies of Ogus *et al*. and Bochud *et al.* show increased susceptibility to tuberculosis in +2477*A carriers.[51,52]
One might hypothesize that inadequate recognition of *Chlamydia* in *TLR2* +2477*A carriers results in increased susceptibility and severity of *Chlamydia* infections.



*TLR9* haplotype combination -1237T/+2848G – -1237C/+2848G reduces susceptibility to *Chlamydia* infections (OR: 0.4). This combination contains the *TLR9* -1237 C allele which is also present in haplotypes 3 and 4, as described above. Ng *et al.* used *in silico* analyses to demonstrate potential differences in NFκB binding affinity between the T and C alleles. They used luciferase and binding assays to demonstrate that the C allele has a higher NFκB binding affinity and results in increased expression of TLR9.[53] One might hypothesize that the increased TLR9 expression in *TLR9* -1237*C carriers increases recognition of *Chlamydia* which results in a reduction of symptoms and late complications of *Chlamydia* infections as observed in our studies and literature.

Previously we have shown that a carrier trait of four genes including TLR4 and TLR9 increases the risk of developing tubal pathology.[37] Other studies have shown the influence of TLR2 and 4 on the pathogenesis of gastritis.[54,55]
This study clIGMearly shows that TLR2 and TLR9 influence the clinical course of *Chlamydia* infections. Specific *TLR2* and *TLR9* haplotypes protect against a more severe clinical course of infection. Alterations in TLR2 function and NFκB binding in TLR9 may explain the association between the genetic variation and the observed differences in *Chlamydia* infections. We did not observe a significant effect for TLR4 in this study. This study provides further evidence for the effect of host genetic variation on *Chlamydia* infections, and

extends our knowledge of the immune system. This may help us define better diagnostic and therapeutic methods in the future.

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