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Chapter 3

TLR2 haplotypes in the susceptibility to and severity of *Chlamydia trachomatis* infections in Dutch women

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Summary

Chlamydia trachomatis infections may cause several disease conditions varying from asymptomatic infections to severe upper genital tract pathology, and thereby causing significant morbidity worldwide. Remarkable interindividual differences in the clinical course of *Chlamydia trachomatis* infection have been observed, and are mainly based on variation in genes encoding immune regulatory, and bacteria sensing proteins. These variations so far have been identified by single nucleotide polymorphisms (SNPs).
As patient-related immunomodulating factors, Toll like receptors (TLRs) are closely involved in pathogen recognition, and host defence in *Chlamydia trachomatis* infections.
The aim of this study is to assess the role of *TLR2* SNPs in genes encoding immune regulatory, and bacteria sensing proteins in the susceptibility to, and severity of *Chlamydia trachomatis* infections.
The study comprised of a STD cohort of 468 women visiting the STD outpatient clinic in Amsterdam, The Netherlands, and a control group of 321 women. The subfertility cohort consisted of 56 women with clinically well-defined tubal pathology visiting the department of Obstetrics and Gynaecology of the Academisch Ziekenhuis Maastricht, The Netherlands, because of subfertility.
The results show no significant differences in *TLR2* genotype frequencies in the susceptibility for *Chlamydia trachomatis* infections between the *Chlamydia trachomatis* positive group and controls. However, haplotype analyses show a significant difference (p: 0.015; OR: 0.28; CI: 0.10 – 0.75) in developing tubal pathology in women with *Chlamydia trachomatis* infection. This suggests that *TLR2* polymorphisms have no effect on the susceptibility to *Chlamydia trachomatis* infections, but certain haplotypes protect against the development of severe late sequelae following *Chlamydia trachomatis* infection. More studies, with larger study populations, are needed to confirm these findings.

Introduction

Chlamydia trachomatis (Ct) is the most important bacterial cause of sexually transmitted infections, and may cause considerable reproductive morbidity with the highest rates in adolescent women. Infection often remains asymptomatic and provides a huge reservoir for transmission of the disease, but identification of these cases by selective screening is difficult and associated costs have to be taken into account when screening programmes are considered.[1,2] Repeated infections seem to be associated with severe upper genital tract pathology including pelvic inflammatory disease (PID), ectopic pregnancy, and tubal infertility, in decreasing order of occurrence.[3] Due to complicated early detection of Ct infection, leading to unrecognized PID, patients may stay untreated. This causes a deteriorated reproductive health of the society, and entails significant expenditures for the treatment of this pathology.

The clinical course of Ct infection shows remarkable interindividual differences in transmission, symptomatic course, persistence or clearance of infection, and development of late complications.

In general, the described differences in clinical course could be explained by the interaction between the host (host factors), pathogen (virulence factors), and environmental factors (such as co-infections). *Chlamydia* consists of different serovars[4,5], but no specific serovars have been identified that clearly link to the course of infection. However, it is clear that bacterial factors are present that influence the course of infection.[6]

Twin studies have advanced the efforts to identify susceptibility genes to infectious diseases. Comparison of concordance rates in monozygotic and dizygotic twins provides an estimate of the size of the genetic component of susceptibility, and for many infectious diseases this is substantial. Recently, Bailey *et al.*[7] published the most relevant study in the field of *Chlamydia* Immunogenetics. They estimated the relative contribution of host genetics to the total variation in lymphoproliferative responses to Ct antigen by analyzing these responses in 64 Gambian pairs of twins from trachoma-endemic areas. Proliferative responses to serovar A EB antigens were estimated in monozygotic and dizygotic twin pairs. They found a stronger correlation and lower within-pair variability in these responses in monozygotic compared to dizygotic twin pairs. The heritability estimate was 0.39, suggesting that host genetic factors contributed almost 40% of the variation.

As patient-related immunomodulating factors, TLRs 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. TLR2 was previously described as the pattern recognition receptor (PRR) for the Ct component peptidoglycan.[8,9]

Functional SNPs in the *TLR2* gene have been described in relation to infection and inflammation[10-12] but no studies were performed before in relation to Ct infections.

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

We have previously shown that SNPs in the *TLR4,* and *TLR9* genes increase the risk of tubal pathology in women with Ct infections[13], but have not yet investigated the role of *TLR2* SNPs in *Ct* pathogenesis.
In knockout mice studies, TLR2 plays an important role as mediator in the innate immune response to Ct infections as well as in the early production of inflammatory mediators and in the development of chronic inflammatory pathology.[14]
We chose two SNPs (+2477, rs5743708 and -16934, rs4696480) that are mainly related to infection. The -16934 polymorphism is associated with sepsis and Gram positive bacteria.[15] Veltkamp *et al.* showed a link between carriage of the A allele and increased production of TNF-α, IL-6, and IL-12.[16] The +2477 polymorphism results in a amino acid substitution at position 753 (Arg753Gln), which has been associated with tuberculosis[17], and has been associated with a reduced responsiveness to *S. aureus* infections.[18]
We aim to assess the role of variation of these two SNPs in Ct positive (Ct+) and Ct negative (Ct-) individuals in general, and Ct+ individuals with or without symptoms. We also aim to analyse inter individual genetic differences in the severity of these infections, as development of tubal pathology post Ct infection.

Materials and methods

Patient populations:

STD cohort
Women of Dutch Caucasian (DC) origin (n = 468), under the age of 33 (ranging 14-33years; median 22 years) and consecutively visiting the STD outpatient clinic in Amsterdam, The Netherlands in the period of July 2001 – December 2004). Ct positive cases (n = 147) were defined as IgG antibody positive (Medac, Wedel, Germany) and Ct DNA positive (Roche Diagnostics, Basel, Switzerland)). Those without Ct infection, based on negative Ct DNA test and negative Ct serology responses, served as controls (n = 321).
One hundred and eighty one women were symptomatic and 287 asymptomatic. Of the symptomatic women 61 were positive and 120 were negative for other microorganisms (*C. albicans, N. gonorrhoeae, T. vaginalis* and *Herpes simplex virus 1/2)*.
One hundred and forty-seven women were Ct+. In this group 66 women had symptoms whereas 81 were asymptomatic. Twenty two women from the symptomatic group were positive and 44 were negative for microorganisms. Of the asymptomatic women 25 patients were positive and 56 were negative for microorganisms. The control group consisted of 321 women.
Participants were asked to sign an informed consent and fill out a questionnaire, regarding their complaints at that moment, varying from increased discharge, having bloody discharge

during and/or after coitus, recent abdominal pain (not gastrointestinal or menses related), and/or dysuria. A cervical swab was taken for the detection of Ct DNA by PCR.[19] Peripheral venous blood was collected for the analysis of IgG antibodies against Ct (Medac Diagnostika mbH, Hamburg, Germany). A titre of ≥1:50 was considered positive. Samples with grey zone values, *e.g.* cut off ± 10%, were repeated, and considered positive when the result was positive, or again within the grey zone. Infections with *Candida albicans, Neisseria gonorrhoeae, 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.[20]

Subfertility cohort

This cohort consisted of DC women (n = 56) with clinically well-defined tubal pathology (TP) visiting the department of Obstetrics and Gynaecology of the Academisch Ziekenhuis Maastricht, The Netherlands, because of subfertility. They were included in the period of December 1990 and November 2000.[21]

A laparoscopy with tubal testing has 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.[22] Subfertile women who had no peri-adnexal adhesions and had patent tubes at laparoscopy served as negative controls. There were 43 (76.8%) patients with TP and 13 (23.2%) without TP. Of 39 Ct+ women 26 had TP. In the Ct- group 17 women had TP.

IgG antibodies to Ct were detected with a species-specific MIF test (AniLabSystems, Finland), as described previously by Land *et al.*[21], with comparable sensitivity and specificity as compared to the IgG ELISA from Medac used for the STD cohort (23). A positive Ct IgG MIF test was defined as a titre ≥1:32.

Immunogenetic analyses

DNA Extraction

STD cohort

Eukaryotic DNA from PBMCs 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.

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

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 (O/N) at 4°C and then stored at -20°C until further analysis.

Subfertility cohort

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

Gene polymorphisms

TLR2 +2477 G>A Arg753Gln (rs5743708) and *TLR2* -16934 T>A (rs4696480) genotyping was performed with the TaqMan method. The primers and probes are described in table 1.

Table 1: primers and probes of *TLR2*

***TLR*2 +2477**	
Forward primer	CATTCCCCAGCGCTTCTG
Reverse primer	TCCAGGTAGGTCTTGGTGTTCAT
Probe for allele A	AAGCTGCAGAAGAT-MGB
Probe for allele G	AAGCTGCGGAAGAT-MGB
***TLR*2-16934**	
Forward primer	TGGTTCTGGAGTCTGGGAAGTC
Reverse primer	CTCACCATGTGATGCTTTCCAT
Probe for allele T	TCTGGTGAGGGTCAT-MGB
Probe for allele A	ATCTGGAGAGGGTCAT-MGB

Statistical analyses

All groups were tested for Hardy-Weinberg equilibrium to check for Mendelian inheritance. Statistical analyses were performed and the Fisher exact and χ^2 tests were used to test for differences in *TLR2* genotype / carrier frequencies between the (sub)groups. P-values <0.05 were considered statistically significant. *TLR2* haplotypes were inferred using PHASE v2.1.1 and SNPHAP.[24-26]

Results

Susceptibility to Ct infection

The overall distributions of the *TLR*2 SNPs (*TLR*2-16934 T>A and *TLR*2 +2477 G>A) are shown in table 2a for the genotypes and in table 2b for the haplotypes. Both on the genotype and the haplotype level, the two analyzed SNPs showed no statistical significance for susceptibility to Ct infections. Subsequently, we analyzed the effect of coinfections with other microorganisms, and symptomatology and found no effect in obtained results.

Thus, there is no evidence that these SNPs predispose the development of Ct infection or coinfection with other microorganisms in established Ct infection.

Table 2a: Susceptibility analyses: Genotypes of the *TLR2 g*ene polymorphisms in subgroups of patients with Ct infection with and without symptoms and controls

TLR2		-16934 T>A						+2477 G>A					
	n	TT	%	TA	%	AA	%	GG	%	GA	%	AA	%
Ct+	147	43	29.3	62	42.2	42	28.6	136	92.5	7	4.8	4	2.7
Ct+ asym	81	28	34.6	31	38.3	22	27.2	78	96.3	0	0.0	3	3.7
Ct+ sym	66	15	22.7	31	47.0	20	30.3	58	87.9	7	20.9	1	1.5
Ct- (Controls)	321	89	27.7	152	47.4	80	24.9	287	89.4	30	9.4	4	1.3

Abbreviations: Ct: *C. trachomatis*, Sym+: symptom positive, CT+: CT DNA & IgG positive; CT-: CT DNA & IgG negative

Tabel 2b: Susceptibility analyses: Frequencies of *TLR2* haplotypes formed by -16934 T >A and +2477 G >A SNPs, in the susceptibility to *Chlamydia* infections

Haplotypes		I		II		III		IV	
	n	TG	%	AG	%	TA	%	AA	%
Ct+	294	135	45.9	144	49.0	13	4.4	2	0.7
Ct+ asym	162	82	50.6	74	45.7	5	3.1	1	0.6
Ct+ sym	132	53	40.2	70	53.0	8	6.1	1	0.8
Ct- (Controls)	642	296	46.1	308	48.0	34	5.3	4	0.6

Abbreviations: Ct: *C. trachomatis*, Sym+: symptom positive, CT+: CT DNA & IgG positive; CT-: CT DNA & IgG negative

Severity of Ct infection

The overall distributions of the *TLR2* SNPs (*TLR*2 -16934 T>A and *TLR*2 +2477 G>A) are shown in table 3a for the genotypes and in table 3b for the haplotypes. The analysed SNPs showed no statistical significant differences among the different groups in the genotype distribution. Haplotype 1 was statistically significant (p: 0.015; OR: 0.28; 95%CI: 0.10-0.75) (figure 1) associated with protection against TP following CT infection. The same haplotype was significantly decreased in increasing severity of Ct infections (asymptomatic > symptomatic > TP; figure 2), suggesting a protective effect of this haplotype against the development late complications.

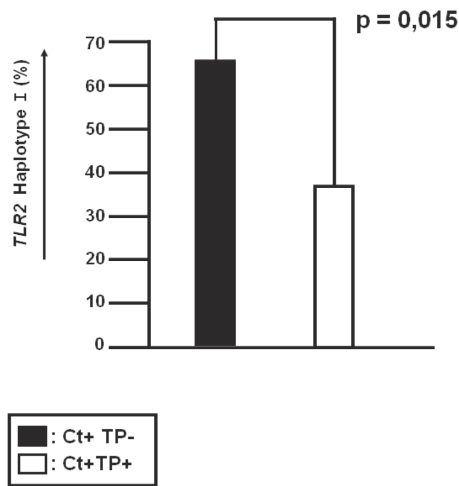

Figure 1: Haplotype I distribution in Ct+ women with and without tubal pathology (p: 0.015; OR: 0.28; 95%CI: 0.10-0.75)

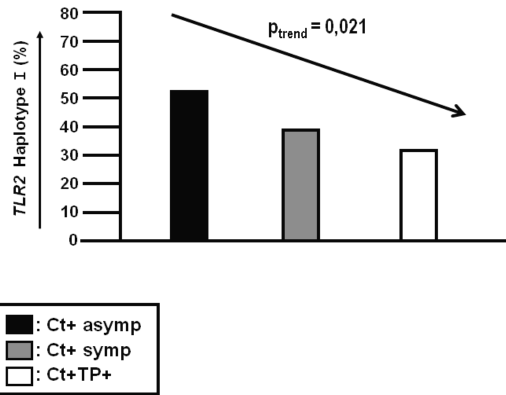

Figure 2: Haplotype I distribution in Ct+ asymptomatic women, Ct+ symptomatic women, and Ct+ TP+ women (p_{trend}: 0.021)

Table 3a: Severity analyses: Genotypes of the *TLR2* gene polymorphisms in subgroups of patients with Ct infection with or without TP

TLR2		-169394 T>A						+2477 G>A					
	n	TT	%	TA	%	AA	%	GG	%	GA	%	AA	%
Ct-TP+	17	6	35.3	8	47.1	3	17.7	16	94.1	1	5.9	0	0.0
Ct+TP-	13	6	46.2	5	38.5	2	15.4	13	100.0	0	0.0	0	0.0
Ct+TP+	26	7	26.9	9	34.6	10	38.5	21	80.8	5	19.2	0	0.0

Abbreviation: CT: C. trachomatis, CT+: CT DNA & IgG positive; CT-: CT DNA & IgG negative.

Table 3b: Severity analyses: Frequencies of *TLR2* haplotypes formed by +2477 G>A and -16934 T>A SNPs

TLR2		I		II		III		IV	
	n	TG	%	AG	%	TA	%	AA	%
Ct-TP+	34	19	55.9	14	41.2	1	3.0	0	0.0
Ct+TP-	26	17	65.4	9	34.6	0	0.0	0	0.0
Ct+TP+	52	18	34.6	29	55.8	5	9.6	0	0.0

Abbreviation: CT: C. trachomatis, CT+: CT DNA & IgG positive; CT-: CT DNA & IgG negative.

Discussion

This study showed no statistically significant differences in genotype distribution in either the susceptibility to or severity of *Chlamydia* infections. However, when the polymorphisms were combined into haplotypes significant associations were observed, showing a protective effect of *TLR2* polymorphisms against the development of complications after Ct infection.

Haplotype I results in a decreased severity of Ct infection. This haplotype contains the wildtype alleles of both studied polymorphisms. The mutant (A) allele of the -16934 polymorphism has been linked to increased production of TNF-α, IL-6, and IL-12[16], although a reduction of IL-6 production has been reported in atopic dermatitis patients.[27] Heterozygous carriage of the +2477 polymorphism has been reported to increase TNF-α production, but also to result in reduced IL-8 and IFN-γ production.[28,29]

Bochud *et al.* observed increased shedding and lesions in genital herpes infections in carriers of two extended *TLR2* haplotypes containing the wildtype alleles of -16934 and +2477.[30] However, other haplotypes containing both wildtype alleles do not show significant differences. It is more likely that the observed effect is caused by other functional polymorphisms within these haplotypes. This is corroborated by other results reported by Bochud *et al.*.[30]

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.[14] 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 found to be tightly associated with the bacteria during the intracellular phase, and that it was recruited to the inclusion membrane together with MyD88. Intracellular TLR2 was suggested to be responsible for the initiation of signal transduction events during infection with Ct.[31]

From the published results and the data from this study, one might hypothesise that the increased production of proinflammatory cytokines observed in carriers of the mutant alleles results in a stronger immune response against Ct, which may then result in collateral damage and thus late complications. This is corroborated by the reduced rate of complications in *TLR2* deficient mice. A similar effect was observed in a previous study where we showed that *CCR5* deficiency results in a delayed clearance of Ct but also in reduced pathology.[32]

It is clear that genetic variation in the host immune system can have an impact on the susceptibility to and severity of Ct infections. Combined carriage of polymorphisms may have a more profound impact of Ct pathogenesis as we have shown previously.[13]

Conclusion

Thus, an adequate recognition of Ct by receptors in the female genital tract is a crucial step in the immune response and may play an important role in the protection of the host against the development of the late complications following Ct infections. To confirm our preliminary data, more studies are needed in larger cohorts. Moreover, with regard to earlier studies, we believe that besides large cohort studies, analysis of carriage of multiple SNPs in the Ct infection related *TLR* pathways may reveal a significant impact on the susceptibility to and severity of *Chlamydia* infections.

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