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ANALYSES OF POLYMORPHISMS IN THE INFLAMMASOME-ASSOCIATED *NLRP3* AND *miRNA-146A* GENES IN THE SUSCEPTIBILITY TO AND TUBAL PATHOLOGY OF *CHLAMYDIA TRACHOMATIS* INFECTION

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SUMMARY

Susceptibility to Chlamydia trachomatis *infections is 40% host based.* microRNA-146a *is a negative regulator of Toll-like receptor (TLR) signaling and possesses functional polymorphisms which decrease the production of pre-miR-146a and mature miR-146a. Single nucleotide polymorphisms (SNPs) in* NLRP3 *are associated with decreased* NLRP3 *expression and hypoproduction of interleukin (IL)-1β. We investigated whether the SNPs* miR-146a *G>C (rs2910164),* NLRP3 *C>T (rs4925663) and G>A (rs12065526) are associated with the susceptibility to and severity of* C. trachomatis *infection. The genotypes of three SNPs were tested in two cohorts: cohort 1 consists of Dutch women (n = 318) attending a sexually transmitted disease (STD) clinic and cohort 2 (n = 277) consists of subfertile (n = 184) and healthy Finnish women (n=93). While in cohort 1 the analyzed SNPs were not associated with the susceptibility to* C. trachomatis *infections (*C. trachomatis*-positive vs.* C. trachomatis*-negative), we showed in* C. trachomatis*-positive women that the NLRP3 mutant AG and AA genotypes were a risk factor for the development of symptoms (P = 0.047, OR = 2.9) and more specifically for having lower abdominal pain (genotype AA: P = 0.022, OR = 31.3). In the Finnish tubal pathology group versus the control group no statistical significant differences in the incidences of the SNPs studied were found, nor for the degree of tubal pathology. In conclusion, the mutant* NLRP3 *A allele is a risk factor for the development of symptoms, specifically lower abdominal pain, after a* C. trachomatis *infection in women attending an STD clinic.*

INTRODUCTION

Chlamydia trachomatis infections, the most prevalent sexually transmitted diseases (STDs), are increasing steadily, with over 90 million new infections annually worldwide (1). In women, over 80% of these infections are asymptomatic. Screening programs have been initiated but are costly and approaches are under debate (2, 3). There are striking differences between individuals in the clinical course and outcome of *C. trachomatis* infection. Some women clear the pathogen without developing tissue damage, whereas others have persistent infection, leading potentially to pelvic inflammatory disease, ectopic pregnancy and tubal infertility. These differences are determined by the interaction between the virulence factors of the pathogen, environmental factors and host immune factors. Bacterial factors including *Chlamydia* serovars, clinical and environmental factors have been studied (4, 5), but these factors are still unable to explain the observed differences in the course of *Chlamydia* infection (6). Recent studies have shown that host genetic variability plays a role in infectious diseases including hepatitis (7) and meningococcal infections (8), in chronic inflammatory diseases and in diseases with both infectious and inflammatory components (9-11). Indeed, in a Gambian twin study as much as 40% of the variance in *C. trachomatis* ocular infections was shown to result from host genetic variations (12). It is reasonable to believe that the genetic variations in important host immune genes may influence the clinical prognosis of *C. trachomatis* infection.

A normal immune response is essential to clear *C. trachomatis* infection. This immune response is initiated by an adequate recognition of the pathogens by the pattern recognition receptors (PRRs) on and in epithelial cells in the genital tract. Different pathogen-associated molecular patterns (PAMPs) of *C. trachomatis* are recognized by the differentially expressed PRRs along the human female genital tract, which include Toll-like receptor 4 (TLR4) and TLR2 through MyD88-dependent pathways (13, 14). These PRRs have been studied in relation to their first-line role in initiation of the innate immune response against *C. trachomatis* (15, 16). The MyD88-dependent branch of the TLR2/4 signaling cascade is initiated and transduced leading to nuclear factor (NF) κB and AP-1 activation and ultimately upregulation of immune-responsive genes (17), which can stimulate human endocervical cells to produce immune active molecules that drive the inflammatory response and recruit classic innate immune cells, resulting in stimulating adaptive immune cells and clearing *C. trachomatis* infection.

TLRs could activate a potent immune response, but the signals transmitted from TLRs must be tightly controlled along the signaling pathway as well, and there is clear evidence that inappropriate activation of TLRs can result in enhanced infection and inflammatory responses and disease (18). This response is influenced by host genetic variations in immune genes, which can result in changes in the function and expression levels of those genes.

The most frequent variation in human genetics is the single nucleotide polymorphism (SNP). We have recently shown that carrying multiple functional SNPs in *C. trachomatis* sensing pathways, such as *TLR4*, *TLR9*, *NOD2* and *CD14* genes, tends to be a risk factor for *C. trachomatis*-related tubal pathology (19). Therefore, further investigation of the genetic regulation of innate immune response is warranted.

NLRP3 (also known as CIAS1) is a component of the NLRP3 inflammasome, which is a multimeric protein complex that mediates the processing of the proinflammatory caspases and cytokines. *NLRP3* expression is primed by NFκB activating PRRs and cytokine receptors, which is mediated by MyD88 through the *NLRP3* promoter (20), and post-transcriptional stimulus such as ATP or crystal-induced damage is required for *NLRP3* activation for assembling NLRP3 inflammasome (21) to positively regulate caspase-1 activity and interleukin (IL)-1β secretion, which is an important inflammatory mediator during inflammation and infection (22, 23). Evidence that NLRP3 inflammasome might be involved in the innate immune response to fungal components has been reported recently (24). The SNPs in *NLRP3* region were found to be implicated in the susceptibility to common inflammatory Crohn's disease (25) and may play a central role in the triggering of vulvar vestibulitis syndrome (VVS) in a subset of *Candida albicans*-infected patients (26).

The *microRNA (miR)-146a* is involved in the regulation of immune responses, among others in the TLR pathways (27). miRNAs are endogenous noncoding RNA molecules of approximately 22 nt in length, as post-transcriptional gene regulators that down-regulate protein expression by translational repression, mRNA cleavage or promotion of mRNA decay depending on the degree of complementarity with the specific target mRNA (28). Sequence variations such as SNPs in the miRNAs and their target sites disrupt the miRNA–mRNA interaction and affect the expression of miRNA targets (29, 30). The G>C polymorphism (rs2910164) has been identified in the *miR-146a* gene. This polymorphism is located in the stem region opposite the mature miR-146a sequence and results in a change from G:U pair to C:U mismatch in the stem structure of miR-146a precursor. The functional SNP reduces the production of pre- and mature miR-146a. The reduction in miR-146a led to less efficient inhibition of target genes involved in the TLR and cytokine signaling pathways (TRAF6, IRAK1) and PTC1, which contributes to the genetic predisposition to papillary thyroid carcinoma (31, 32) and hepatocellular carcinoma (33). Figure 1 shows the Ingenuity-based pathway, including both miRNA-146a and NALP3.

Therefore, the aim of our study was to assess whether the polymorphisms in the *miRNA-146a* and *NLRP3* genes are associated with susceptibility to and severity of *C. trachomatis* infections using an STD cohort and a subfertility cohort with a control group.

MATERIALS AND METHODS

Patient populations

STD cohort (cohort 1)

Dutch Caucasian women (n = 318), under the age of 32 years (range 15–32 years; median 22 years) visiting the STD outpatient clinic in Amsterdam, the Netherlands between July 2001 and December 2004, were included in this study. Questionnaire-based symptoms and clinical coinfection data were collected (34). Those with co-infections (*C. albicans, Neisseria gonorrhoeae*, *Trichomonas vaginalis*, herpes simplex virus 1 and 2) were excluded from the study since symptoms could be related to those coinfections. From the 318 women without coinfection, 99 were defined as cases (*C. trachomatis* IgG antibody [Medac, Wedel, Germany] and *C. trachomatis* DNA-positive [Roche Diagnostics, Basel, Switzerland]) and 219 women were defined as controls (*C. trachomatis* IgG antibody [Medac, Wedel, Germany] and *C. trachomatis* DNA-negative [Roche Diagnostics, Basel, Switzerland]) (35). Of all the participants, 119 had symptoms, including 47 women with lower abdominal pain; 74 women with abnormal secretion; 28 women with dysuria; and 17 women reporting bleeding during or after coitus (34).

Finnish subfertility cohort (Cohort 2)

Finnish Caucasian infertile women (n = 184), with detected prior *C. trachomatis* infection, who had attended the In Vitro Fertilization Unit, Department of Obstetrics and Gynecology, Helsinki University Hospital, Helsinki, Finland from 1990–2005 (described previously; 36) participated in a severity study. A history of previous infection with *C. trachomatis* was evaluated by measuring *C. trachomatis* response (CTR)-specific humoral and cell-mediated immunity, as described previously (37, 38). All infertile women had at least one positive immunologic marker of past *C. trachomatis* infection. Of all of them, 63 cases (34%) with tubal factor infertility (TFI) confirmed by laparoscopy were classified into three subgroups according to the classification of Hull and Rutherford (39). Minor damage (tubal pathology 1; TP1) was found in 12 of 63 cases; moderate damage (TP2) in 31 cases; and severe damage (TP3) in 20 cases. As *C. trachomatis* infection controls, 121 (66%) women without TFI verified by laparoscopy were participating in the present study. The healthy controls consisted of 93 female blood donors without *C. trachomatis* infection.

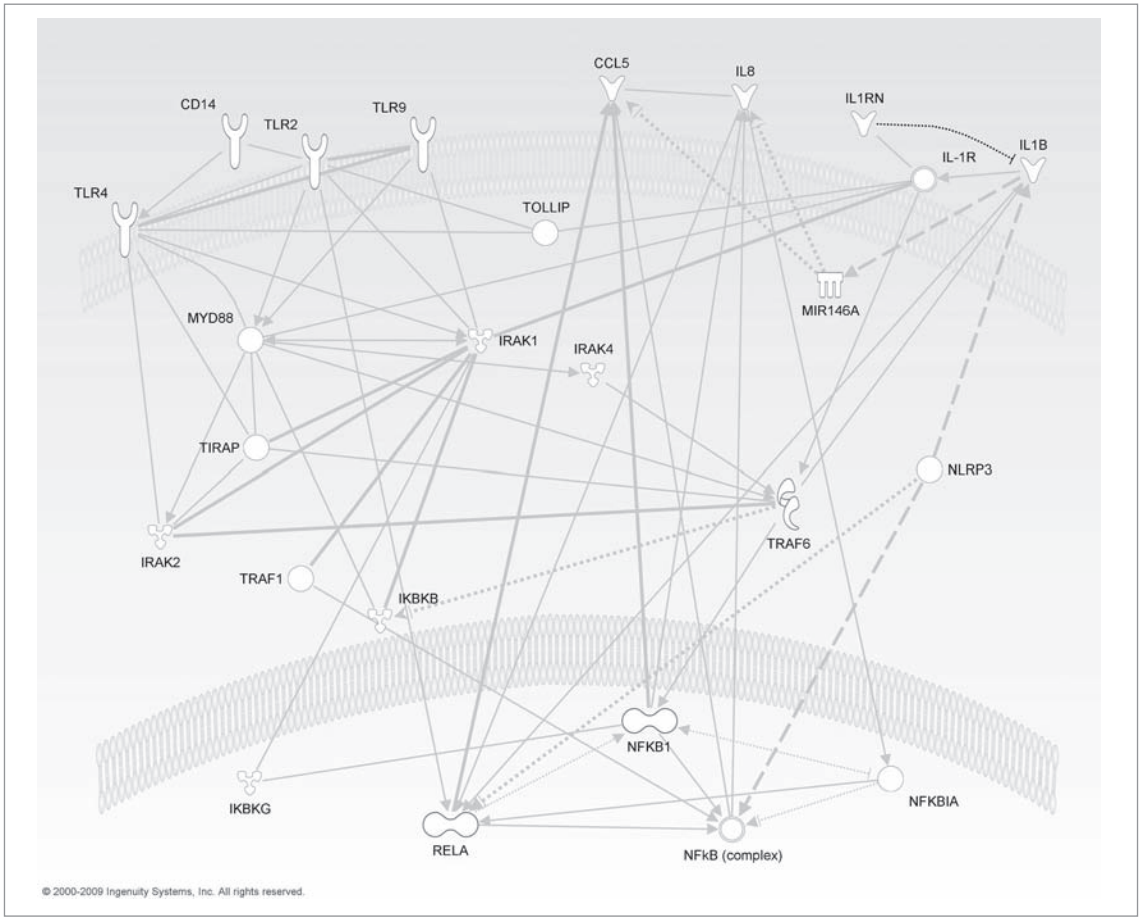

Figure 1. Pathway representation of mi-RNA-146a and NLRP3 in innate immunity. Partial overview of pathway interaction involved in immune regulation constructed using Ingenuity Systems software. Depicted are pathogen recognition receptors (e.g., Toll-like receptors; TLRs), regulatory cytokines (e.g., interleukin-1β; IL-1β), signal transducing proteins in the pathway, and regulators of the pathway (e.g., NALP3 and miRNA-146). The lines indicate interactions between the different molecules. Connecting lines without arrow heads indicate binding. Lines with arrowheads indicate activation or increased expression. Dashed lines indicate indirect interaction (e.g., the end result may be increased expression, but the effect is mediated through proteins not shown in this figure). Dotted lines indicate inhibition. This figure is for overview purposes only, and does not represent the full range of pathways and interactions in the immune system.

Immunogenetic analysis

Genomic DNA was isolated according to the methods described by Ouburg et al. (34) and Ohman et al. (36). Oligonucleotide primer and probe sets of *miR-146a* were designed, primer: GAA CTG AAT TCC ATG GGT TGT GT (forward) and GCC CAC GAT GAC AGA GAT ATC C (reverse); probes: miR-146aC: VIC-ACC TCT GAA ATT CA and miR-146aG: 6FAM-AGA CCT GTG AAA TTC AGT T (ABI, Warrington Cheshire, UK); that of *NLRP3* were designed based on TaqMan™ probe primer combinations available from the Assay-on-Demand™ human SNP genotyping collection from Applied Biosystems Incorporated (rs Rs12065526 Assay on demand C__30713870_10 (ABI, Foster City, CA, USA); rs Rs4925663 Assay on demand C__26052022_10 (ABI, Foster City, CA, USA). Genotyping was performed using standard Taqman protocols. The SNPs studied are summarized in Table I.

Table I. *The single nucleotide polymorphisms (SNPs) studied.*

Gene	SNP	Number	Location	Gene function with innate immune*
NLRP3	G>A	rs12065526	1q44	Negative regulation of i) *NFκB* transcription activity and ii) import to nucleus; positive regulation of caspase-1 activity and IL-1β secretion
NLRP3	C>T	rs4925663	1q44	
miR-146a	G>C	rs2910164	5q33.3	Negative regulation of severe inflammation during the innate immune response

miR-146a, Homo sapiens microRNA-146a, *Hsa-miR-146a*; *NLRP3*, NOD-like receptor family, pyrin domain containing 3;
*For gene function refer to http://www.ncbi.nlm.nih.gov/gene/

Statistical analyses

All groups were tested for Hardy-Weinberg equilibrium to check for Mendelian inheritance. Statistical analyses were performed using Instat GraphPad and SPSS version 11 (SPSS Inc., Chicago, IL, USA). For the susceptibility cohort, the following analyses were performed: i) positive cases as *C. trachomatis* infection controls versus *C. trachomatis* infection controls as negative controls; ii) for the cases, women with versus those without symptoms (if found positive, specific symptoms were introduced in the analysis). For the subfertility cohort, the following analyses were performed: i) comparison between *C. trachomatis*-positive women with and without tubal pathology and compared with controls; and ii) SNP incidences comparisons between women with different degrees of tubal pathology. Fisher exact and chi-square tests were used to test for differences in three SNPs allele or genotype frequencies between the subgroups. P-values < 0.05 were considered statistically significant.

RESULTS

All genotypes distributions of *miR-146a* G>C (rs2910164), *NLRP3* C>T (rs4925663) and G>A (rs12065526) tested were in Hardy-Weinberg Equilibrium in the STD and subfertility cohorts.

Genotype distribution in the susceptibility to *C. trachomatis* infection (Cohort 1)

Table II shows the genotype distributions determined for *miR-146a* G>C (rs2910164), *NLRP3* C>T (rs4925663) and *NLRP3* G>A (rs12065526). While in cohort 1 the three analyzed SNPs were not associated with the susceptibility to *C. trachomatis* infections (*C. trachomatis*-positive vs. *C. trachomatis*-negative), we showed in *C. trachomatis*-positive women carrying the mutant AG and AA genotypes were at risk for the development of symptoms (P = 0.047, OR = 2.9, 95% CI [1.0–8.0]). More specifically, the symptom significantly associated with symptoms was lower abdominal pain (genotype AA: P = 0.022, OR=31.3, 95% CI [1.4-689]).

Genotype distribution in the severity of sequelae to *C. trachomatis* infection

Table III shows the genotype distributions determined for *miR-146a* G>C (rs2910164), *NLRP3* C>T (rs4925663) and *NLRP3* G>A (rs12065526). In the Finnish tubal pathology group versus the control group, no statistical significant differences in the incidences of the SNP studied were found, nor for the degree of tubal pathology.

DISCUSSION

Over recent decades, immunogenetic studies have provided insight into the individual differences in the pathogenesis of infectious diseases and a recent study showed that 40% of the susceptibility to *C. trachomatis* infection is based on host genetic factors (12). In the current study we investigated whether SNPs in *miR-146a* and *NLRP3* genes which regulate TLR signaling pathway and cytokines, respectively, might be involved in host innate immune response.

We showed in our STD cohort that the three analyzed SNPs in *NLRP3* and *miR-146a* were not associated with the susceptibility to *C. trachomatis* infections (*C. trachomatis*-positive versus *C. trachomatis*-negative). However, we showed in the same cohort that *C. trachomatis*-positive women carrying the mutant A allele were at risk for the development of symptoms (P = 0.045) with lower abdominal pain as the specific symptom (P = 0.022). These data suggest that the regulation of *NLRP3* expression may play a role in the symptomatology of an uncomplicated *C. trachomatis* infection. In the second cohort focusing on tubal pathology, no statistically

Table II. *Genotype distribution in sexually transmitted diseases and C. trachomatis susceptibility cohort 1.*

			miR-146a (rs2910164)			*NLRP3* (rs4925663)			*NLRP3* (rs12065526)		
	CT groups	Total	1.1(GG) n (%)	1.2(GC) n (%)	2.2(CC) n (%)	1.1(CC) n (%)	1.2(CT) n (%)	2.2(TT) n (%)	1.1(GG) n (%)	1.2(GA) n (%)	2.2(AA) n (%)
CT+	Cases	99	49 (49.5)	44 (44.4)	6 (6.1)	36 (36.4)	51 (51.5)	12 (12.1)	79 (79.8)	18 (18.2)	2 (2.0)
	Sym+	44	21 (44.7)	19 (43.2)	4 (9.1)	18 (40.9)	21 (47.7)	5 (11.4)	31 (70.4)	11 (25)	2 (4.5)[a]
	Sym–	55	28 (50.9)	25 (45.5)	2 (3.6)	18 (32.7)	30 (54.5)	7 (12.7)	48 (87.3)	7 (12.7)	0 (0.0)[a]
	LAP+	15	6 (40)	7 (46.7)	2 (13.3)	5 (33.3)	9 (60)	1 (6.7)	10 (66.7)	3 (20.0)	2 (13.3)[b]
	LAP–	84	43 (52.2)	37 (44.0)	4 (4.8)	31 (36.9)	42 (50)	11 (13.1)	69 (82.1)	15 (17.9)	0 (0.0)[b]
CT–	Controls	219	117 (53.4)	89 (40.16)	13 (5.9)	75 (34.2)	111 (50.7)	33 (15.1)	156 (71.2)	60 (27.4)	3 (1.4)
	Sym+	75	40 (53.3)	31 (41.3)	4 (5.3)	30 (40.0)	35 (46.7)	10 (13.3)	55 (73.3)	20 (26.7)	0 (0.0)
	Sym–	144	77 (53.5)	58 (40.3)	9 (6.2)	45 (31.2)	76 (52.8)	23 (15.9)	101 (70.1)	40 (27.8)	3 (2.1)
	LAP+	32	15 (46.9)	15 (46.9)	2 (6.2)	11 (34.4)	17 (53.1)	4 (12.5)	21 (65.5)	11 (34.4)	0 (0.0)
	LAP–	187	102 (54.5)	74 (39.6)	11 (5.9)	64 (34.2)	94 (50.3)	29 (15.5)	135 (72.2)	49 (26.2)	3 (1.6)

CT, *Chlamydia trachomatis;* Sym+, symptom positive; LAP+, lower abdominal pain positive; CT+, CT DNA and IgG-positive; CT–, CT DNA and IgG-negative. [a]GG vs. GA + AA in CT-positive subjects with symptoms vs. CT-positive subjects without symptoms; P = 0.047, OR = 2.9. [b]GG + GA vs. AA in CT positives with LAP vs. without LAP; P = 0.022, OR = 31.3.

significant associations were found, including for the degree of tubal pathology. Interestingly, we also tested (data not shown due to small sample size) a Dutch Caucasian group of subfertile *C. trachomatis* IgG-positive women (N = 37), who visited the Department of Obstetrics and Gynecology of the Academisch Ziekenhuis Maastricht, the Netherlands, previously described by Den Hartog et al. (19). Of the 37 women, 27 had laparoscopically confirmed severe tubal pathology (cases) and 12 had no tubal pathology (controls). The three SNPs analyzed had the same incidence as in the Finnish cohort, and thus no associations were found for an enhanced risk to develop tubal pathology, confirming the data obtained in the Finnish population.

During *C. trachomatis* infection host immune response can evoke *NLRP3* expression against *Chlamydia* via the

Table III. *Genotype distribution in subfertility and C. trachomatis severity cohort 2.*

			miR-146a (rs2910164)			*NLRP3* (rs4925663)			*NLRP3* (rs12065526)		
CT status	Subgroup	Total	1.1(GG) n (%)	1.2(GC) n (%)	2.2(CC) n (%)	1.1(CC) n (%)	1.2(CT) n (%)	2.2(TT) n (%)	1.1(GG) n (%)	1.2(GA) n (%)	2.2(AA) n (%)
IgG+	TP+	63	35 (55.6)	26 (41.3)	2 (3.2)	19 (30.1)	34 (54)	10 (15.9)	52 (81)	11 (17.4)	1 (1.6)
	TP–	121	64 (529)	48 (39.7)	9 (7.4)	39 (32.2)	63 (52.1)	19 (15.7)	93 (76.8)	26 (21.5)	2 (1.7)
	TP–	93	55 (59.1)	33 (35.5)	5 (5.4)	24 (25.8)	52 (55.9)	17 (18.3)	66 (71.0)	27 (29.0)	0 (0)
IgG–	Tubal damage	63									
	TP-1	12	6 (50)	6 (50)	0 (0)	1 (8.3)	10 (83.4)	1 (8.3)	10 (83.3)	2 (16.7)	0 (0)
	TP-2	31	17 (54.8)	14 (45.2)	0 (0)	11 (35.5)	15 (48.4)	5 (16.1)	27 (87.1)	4 (12.9)	0 (0)
	TP-3	20	12 (60)	6 (30)	2 (10)	7 (35)	9 (45)	4 (20)	14 (70)	5 (25)	1 (5)

Distribution in the total cohort, subdivided in *C. trachomatis* IgG-positive women with or without tubal pathology. CT, *Chlamydia trachomatis;* TP, tubal pathology. Severity of tubal damage is classified into TP-1, TP-2 and TP-3, as defined by the classification of Hull and Rutherford (39).

proinflammatory cytokine IL-1β. Recently, genetic variants located in the *NLRP3* promoter (20) and downstream region (40) were identified in patients with inflammatory diseases. This suggests that dysregulated *NLRP3* expression could evoke inflammatory-related diseases. The two SNPs selected are just upstream of the 5.3 kb predicted regulatory region associated with Crohn's disease, in which the homozygosity for the risk allele is associated with the decreased level of *NLRP3* expression and hypoproduction of IL-1β under lipopolysaccharide (LPS)-stimulated conditions (40). These two SNPs tested were not in linkage disequilibrium with all risk SNPs in 5.3 kb *NLRP3* downstream region; however this does not exclude the same biological mechanism of inflammation. Recent clinical data have indicated that *C. trachomatis*-positive symptomatic women show significantly higher chlamydial load compared with *C. trachomatis*-positive women with fertile disorder. IL-1β levels show a significant correlation (R = 0.77) with chlamydial load in symptomatic women. In these symptomatic cases, a persistently high chlamydial load may lead to increased secretion of inflammatory cytokines, thus leading to acute inflammation (41). Taken together, we speculate that the *NLRP3* G>A SNP (rs12065526) might take part in the regulation of *NLRP3* expression and exert a role in the symptomatology of *C. trachomatis* infection.

miR-146a fine-tunes signaling of TLRs and cytokines through negative regulation involving miR-146a targeted IRAK-1 and TRAF6 post-transcriptional repression (27). The negative regulation of IL-1β-induced release of chemokines is only seen at high IL-1β concentrations, which indicates that *miR-146a* might be an important feedback mechanism during severe inflammation in the innate immune response (42). Evidence also showed that there were notable differential IL-1β levels during *C. trachomatis* disease expression (41).

Persistent and recurrent *C. trachomatis* infection may lead to severe late complications (43-45). Recent studies show that most women with fertile disorder have a 100-fold lower chlamydial load in the cervix than that of most of the symptomatic women (41). The consequences could be that *Chlamydia* will be inadequately removed from the infected cells, resulting in reduced atypical, metabolically less active *C. trachomatis* replication and persistence, which may prime low-grade immune response to continuous elaborated *Chlamydia* stress-response proteins (41, 46), and activating different gene transcription profiles to cause continuous secretion of inflammatory cytokines, ultimately leading to late complications such as infertility (47).

Although IL-1β concentration was higher in *C. trachomatis*-infected women with fertile disorder than that in symptomatic and asymptomatic women, chlamydial load in women with fertile disorder only showed a significantly positive correlation with IL-10 levels and not with IL-1β (41). Higher secretion of IL-10 might counteract the higher secretion of interferon (IFN)-γ for clearance of infection. Therefore, higher levels of inflammatory cytokines might restrict chlamydial replication, leading to their persistence (41). When polymorphisms in specific IL-1β and IL-1 receptor antagonist genes were studied, no association with the risk of tubal pathology was found (48). Taken together, we speculate that the interaction of the persistent *C. trachomatis* infection and host innate immune cells might affect the polarization of host common and pathogen-specific immune response, which might explain that the *miR-146a* G>C (rs2910164), *NLRP3* C>T (rs4925663) and G>A (rs12065526) polymorphisms may not severely impact the development of tubal pathology.

CONCLUSIONS

From our results it can be concluded that *NLRP3* G>A (rs12065526) might be associated with a symptomatic course in women with uncomplicated *C. trachomatis* infection, an association which needs to be studied further. The association with NALP3 did not hold in women with tubal pathology as shown in our Finnish and small Dutch Caucasian cohort, emphasizing potential different immunological mechanisms.

Large genetic epidemiological studies are needed to study the association between genetic variation in the host immune system and *C. trachomatis* infection to unravel the complex immunopathogenesis of *C. trachomatis* infection. These studies should be based on well-defined *C. trachomatis* PAMPs, PRR signaling pathways, and the host innate and adaptive immune system to obtain functional polygenic information.

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DISCLOSURE

The authors have nothing to disclose.

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