Chlamydia trachomatis
Identifying women with tubal factor subfertility

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Contents

ABBREVIATIONS

APC	antigen-presenting cell
bp	base pair
BSA	bovine serum albumin
C.	*Chlamydia*
CARD	caspase recruitment domain
CAT	*Chlamydia* IgG antibody testing
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
cHSP	chlamydia heat shock protein
CI	confidence interval
Cpn	*C. pneumoniae*
Cps	*C. psittaci*
Ctr	*C. trachomatis*
DAB	diaminobenzidine
DTP	distal tubal pathology
EB	elementary body
ELISA	enzyme-linked immunosorbent assay
HC	healthy controls
HEp	human epithelial
hHSP	human heat shock protein
hs-CRP	high-sensitivity C-reactive protein
HSG	hysterosalpingography
IFN	interferon
IFU	inclusion-forming units
Ig	immunoglobulin
IL	interleukin
int. conc.	interval conception
KO	knockout
LCR	ligase chain reaction
LGV	lymphogranuloma venereum
LPB	LPS-binding protein
LPS	lipopolysaccharide
LS	laparoscopy

mCD14	membrane-bound cluster of differentiation 14
MIF	micro-immunofluorescence
MOMP	major outer membrane protein
N.	*Neisseria*
NA	not analyzed
NAAT	nucleic acid amplification test
NF	nuclear factor
NICE	National Institute for Clinical Excellence
NOD	nucleotide-binding oligomerization domain
NPV	negative predictive value
OR	odds ratio
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PID	pelvic inflammatory disease
PPV	positive predictive value
PRR	pattern recognition receptor
rASRM	revised American Society for Reproductive Medicine
RB	reticulate body
RFLP	restriction fragment length polymorphism
sCD14	soluble cluster of differentiation 14
SNP	single nucleotide polymorphism
STD	sexually transmitted disease
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
TP	tubal pathology
WT	wild-type

Chapter 1
General introduction

Adapted from:
Chlamydia trachomatis-associated tubal factor subfertility: immunogenetic aspects and serological screening
JE den Hartog, SA Morré, JA Land
Human Reproduction Update 2006, 12 (6): 719-730

INTRODUCTION

History and taxonomy

After analysis of medical papyri, ancient depictions and mummy autopsy data, it was suggested that the ocular disease currently known as trachoma had been endemically present, as *nehat* disease, in Egypt since ancient times (Nunn, 1996). It took until 1957 to isolate the micro-organism *Chlamydia* (*C.*) *trachomatis* for the first time (Wang, 1999). The causal relationship between *C. trachomatis* and both trachoma and genital tract infection was established in 1958 (Collier *et al.*, 1958). Resemblances in microscopic characteristics between trachoma and psittacosis (later identified as caused by *C. psittaci*) were already noticed in the early 1930s by an ophthalmologist (Thygeson, 1934). Gene sequence studies resulted in the discovery of *C. pneumoniae* at the end of the 1980s (Grayston, 1989). New molecular diagnostic methods ultimately led to a new taxonomy of the order of *Chlamydiales*, splitting the family of *Chlamydiaceae* into two genera: *Chlamydia* (with amongst others the species *C. trachomatis*) and *Chlamydophila* (Table I) (Everett *et al.*, 1999, Bush and Everett, 2001).

Characteristics of *Chlamydiaceae*

Chlamydiaceae are obligate intracellular pathogens, which are unable to replicate outside a host cell. *Chlamydiaceae* have a unique biphasic developmental cycle (Figure I). The extracellular forms, elementary bodies (EBs), are infectious and metabolically inactive. After being endocytosed by host cells, they group into intracytoplasmic vacuoles (referred to as inclusions) within 12 to 24 hours. Subsequently, EBs transform into non-infectious but metabolically active and replicating reticulate bodies (RBs). Two or more days after host cell infection, RBs convert back into EBs, followed by their release through cell rupture or fusion of the inclusion with the host cell membrane.
C. trachomatis is subdivided in three human biovars. The trachoma biovar consists of serovars A to C, which are mainly associated with trachoma, but with genital tract infections and reactive arthritis as well. The oculogenital biovar consists of serovars D to K, which are associated with genital tract infections, neonatal and adult conjunctivitis, respiratory tract infections and reactive arthritis. The lymphogranuloma venereum (LGV) biovar consists of serovars L1 to L3, causing infections of the inguinal lymph nodes.

Table I. Taxonomy of the order of *Chlamydiales* (adapted from Bush and Everett, 2001).

Order	Family	Genus	Species	Typical host	Disease
Chlamydiales	*Chlamydiaceae*	*Chlamydophila*	*C. abortus*	Mammals (sheep, cattle, goats)	Abortion
			C. psittaci	Birds	Ocular infection Respiratory tract infection Gastro-intestinal infection
			C. felis	Cats	Ocular infection Respiratory tract infection
			C. caviae	Guinea pigs	Ocular infection
			C. pecorum	Mammals (sheep, cattle, goats, koalas, swine)	Ocular infection Respiratory tract infection Urogenital tract infection Abortion
			C. pneumoniae	Humans	Respiratory tract infection Atherosclerosis Alzheimer's disease
		Chlamydia	*C. trachomatis*	Humans	Ocular infection Genital tract infection Lymphogranuloma venereum Reactive arthritis
			C. suis	Pigs	Intestinal infection
			C. muridarum	Mice, hamsters	Genital tract infection
	Parachlamydiaceae		*P. acanthamoebae*	Amoebae	Respiratory tract infection
			N. hartmannellae	Amoebae	Ocular infection
	Waddliaceae		*W.chondrophila*	Cows	Abortion
	Simkaniaceae		*S. nevegensis*	Humans	Respiratory tract infection

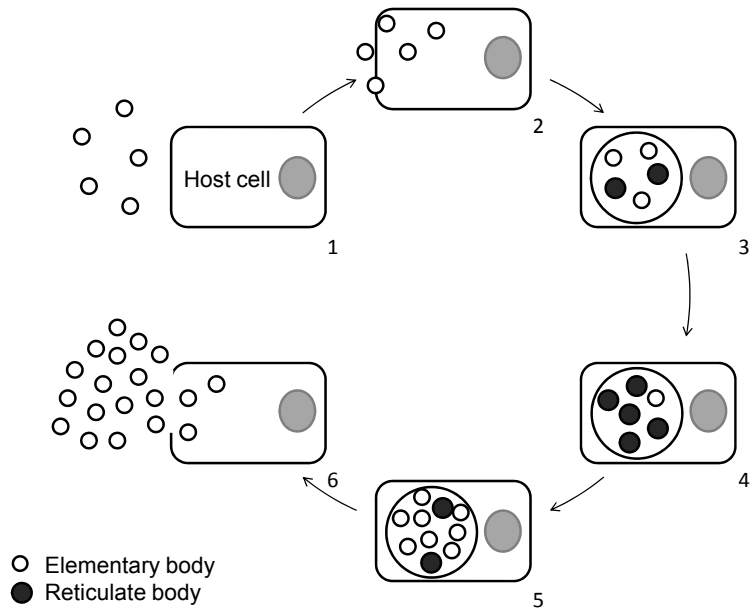

Figure I. Developmental cycle of *C. trachomatis*.
Elementary bodies (EBs) approach the host cell (1) and enter the host cell by endocytosis (2). EBs group into an inclusion and transform into reticulate bodies (RBs) (3). RBs replicate (4) and convert back into EBs (5), which are released from the host cell (6).

Clinical introduction

C. trachomatis is a sexually transmitted pathogen primarily targeting columnar epithelial cells of the cervix. In the Netherlands, an estimated 35 000 cases of *C. trachomatis* cervicitis occur annually (Health Council of The Netherlands, 2004). Symptoms of *C. trachomatis* cervicitis are vaginal discharge, irregular or post-coital bleeding and dysuria. However, up to 70-80% of the *C. trachomatis* infections in women are asymptomatic, and therefore remain unrecognized and untreated (Rahm *et al.*, 1988). A normal immune response (rather than antibiotic treatment) is therefore essential to clear the pathogen and to protect women from ascendance of the infection to the upper genital tract and/or transmission to a sexual partner. Studies on the natural course of untreated *C. trachomatis* lower genital tract infections in women show spontaneous clearance rates of ~ 20-30% in the first weeks to months, ~ 50% in one year, ~ 80% in two years and ~ 94% in four years (review by Golden *et al.*, 2000, Joyner *et al.*, 2002, Morré *et al.*, 2002, Molano *et al.*, 2005, Geisler *et al.*, 2008). Although these studies suggest that the majority of infected women seems to have an adequate local immune response, a subset of infected

women will have a long-lasting *C. trachomatis* infection and therefore an increased risk of ascendance and late sequelae. The above-mentioned studies on spontaneous clearance of *C. trachomatis* lower genital tract infections may even underestimate the percentage of women at risk of complications, since clearance from the lower genital tract does not necessarily mean that the infection has not already ascended to the upper genital tract. Given a worldwide prevalence of 50 million new *C. trachomatis* infections in women each year, a clinically significant group of infected women will be at risk of late sequelae (World Health Organization, 2001).

If a cervical *C. trachomatis* infection is not cleared adequately, the infection may ascend to the endometrium, tubes, pelvis and peri-hepatic region (due to spread of the micro-organism via the paracolic gutters to the liver capsule) and lead to pelvic inflammatory disease (PID). The estimated prevalence of *C. trachomatis* PID in the Netherlands is 5 000—10 000 cases each year (Health Council of The Netherlands, 2004). PID can cause low-grade fever, vaginal discharge and abdominal complaints, but often gives rise to virtually no or only non-specific symptoms.

In 30-40% of women with cervicitis, histological evidence of endometritis has been found (Paavonen *et al.*, 1985a, Wiesenfeld *et al.*, 2002). The micro-organism itself has been isolated from the endometrium in one-third of women with a *C. trachomatis* cervicitis and/or urethritis (Jones *et al.*, 1986). In 70% of women with suspected PID, histological evidence of endometritis has been demonstrated (Paavonen *et al.*, 1985b). Salpingitis has been demonstrated in 10% of women with endometritis (Cates and Wasserheit, 1991). In patients with tubal factor subfertility or ectopic pregnancy, the mean detection rate of *C. trachomatis* in tubal tissue is 11% (range 0—70%) (Osser and Persson, 1992, Lan *et al.*, 1995, Haeusler *et al.*, 1997, Gérard *et al.*, 1998, Hinton *et al.*, 2000, Barlow *et al.*, 2001, Noguchi *et al.*, 2002, Debattista *et al.*, 2004, Bjartling *et al.*, 2007). In the Netherlands, *C. trachomatis* has been estimated to be responsible for tubal factor subfertility in 1 000-2 000 women each year and for 200-400 cases of ectopic pregnancies, which is half of the total yearly number of ectopic pregnancies (Health Council of The Netherlands, 2004). Tubal pathology accounts for 20-25% of the cases of subfertility in developed countries (Collins *et al.*, 1995, Collins and Van Steirteghem, 2004) and for up to 80% of the cases of subfertility in developing countries (Collet *et al.*, 1988).

The pathogenesis of *C. trachomatis*-associated tubal pathology is not yet fully understood. Two mechanisms are assumed to be responsible for the development of tubal damage. The first and probably most important mechanism is by a persistent infection causing a chronic low-grade immune response, which attacks and destroys the host cells (i.e. epithelial cells of the distal part of the fallopian tubes) (LaVerda *et al.*, 1999). Secondly, *C. trachomatis* itself can damage the host tubal epithelial cells when its replication cycle has been completed and elementary bodies are released by cytolysis of the host cell. The latter mechanism does not appear to play a major role in persistent infections, since persistence is characterized by reduced replica-

tion of the dormant pathogen (AbdelRahman and Belland, 2005, Mpiga and Ravaoarinoro, 2006). These aberrant persisting chlamydia particles have been identified in the genital tract, while previously these aberrant forms were only visualized in cell culture under special conditions (Bragina *et al.*, 2001). More studies are needed to elucidate the precise immunopathogenesis of *C. trachomatis* infections.

SCREENING FOR *C. TRACHOMATIS*-ASSOCIATED TUBAL FACTOR SUBFERTILITY

History

Since the majority of *C. trachomatis* infections remains asymptomatic, a patient's history will usually not be helpful in assessing the risk of a previous *C. trachomatis* infection and tubal factor subfertility (Rahm *et al.*, 1988, Logan *et al.*, 2003, Coppus *et al.*, 2007). Conversely, a history of (suspected) previous genital tract infection is not necessarily associated with tubal pathology or subfertility (Hubacher *et al.*, 2004, Andersen *et al.*, 2005). In a cohort of 321 subfertile women undergoing laparoscopy with tubal testing, no correlation has been found between a history of symptoms associated with a possible previous upper genital tract infection (prolonged abdominal pain, dyspareunia or pain during pelvic examination, which was reported by 48% of participants) or a history of symptoms associated with a possible previous lower genital tract infection (vaginal discharge, which was reported by 72% of participants) and the presence of tubal pathology at laparoscopy (Hubacher *et al.*, 2004). A large follow-up study, which has evaluated the risk of subfertility following a positive *C. trachomatis* test on samples obtained from the cervix and/or urethra, has shown that birth rates and time to birth were comparable between women testing positive and negative respectively, although it should be noted that nearly all positive cases in this study received antibiotic treatment (Andersen *et al.*, 2005). The risk of subfertility following untreated *C. trachomatis* lower genital tract infections is therefore assumed to be higher. If a patient's history mentions a PID, the risk of tubal pathology is increased (Weström, 1980, Weström *et al.*, 1992, review by Luttjeboer *et al.*, 2009), although it should be noted that in ~ 30% of women presenting with abdominal pain and clinical signs of PID, immediate laparoscopic findings do not support this diagnosis (Bevan *et al.*, 1995). A clear correlation has been demonstrated between both the number of episodes and severity of laparoscopically verified PID and the risk of tubal disease (Weström, 1980, Weström *et al.*, 1992). In a large follow-up study in women with laparoscopically verified PID diagnosed between 1960 and 1984, the risk of tubal factor subfertility was about 10% after one episode of PID, 20% after two episodes and 40% after three episodes (Table II). *C. trachomatis* accounted for nearly 40% of all PIDs in this study, although

routine *C. trachomatis* testing was introduced in the authors' clinic only in 1977 and was therefore not applied in all PID cases (Weström *et al.*, 1992). The incidence of tubal factor subfertility increased significantly with the severity of PID at laparoscopy, and was 0.6% after one mild episode, 6% after one moderately severe episode and 21% after one severe episode of PID (Weström *et al.*, 1992) (Table II).

Table II. The number of episodes and severity of laparoscopically verified pelvic inflammatory disease (PID) in relation to the risk of tubal factor subfertility (TFS) (adapted from Weström *et al.*, 1992).

No. of episodes of PID	Severity of PID	n (%)	Risk of TFS (%)
One	Mild	312 (25)	0.6
	Moderate	450 (36)	6.2
	Severe	229 (18)	21.4
	All grades	991 (80)	8.0
Two		185 (15)	19.5
Three or more		65 (5)	40.0
TOTAL		1241 (100)	11.4

Several test methods are available to assess the risk of *C. trachomatis*-associated tubal pathology in subfertile women. The reference standard for diagnosing tubal pathology in subfertile women is laparoscopy with tubal dye testing, by which tubal patency and the presence of peri-adnexal adhesions can be assessed. However, laparoscopy has several disadvantages. First, it is an invasive and expensive procedure (in the Netherlands about 1 000 euros) (Fiddelers *et al.*, 2004), requiring general anaesthesia. Operating facilities may not be easily available in every clinic. Furthermore, it holds a 1.5% risk of surgical complications (e.g. bleeding, infection) (Chapron *et al.*, 1998). Owing to these disadvantages, laparoscopy with tubal dye testing is unsuitable as a screening procedure in subfertile women on a large scale. It would be preferable to estimate the risk of tubal pathology before laparocopy, in order to select only high-risk patients for this procedure. Two frequently used screening methods for assessing the risk of tubal pathology are hysterosalpingography (HSG) and serological testing.

Hysterosalpingography

Today, HSG is a widely used screening method for evaluating tubal patency in subfertile women. Compared to laparoscopy with tubal testing, HSG is less expensive (in the Netherlands about 150 euros) (Fiddelers *et al.*, 2004). Furthermore, an advantage of HSG is that tubal flushing using oil-soluble contrast medium has a positive effect on pregnancy rates (Luttjeboer *et al.*, 2007). However, HSG is less accurate in diagnosing tubal pathology as compared to laparoscopy with tubal testing.

HSG has a sensitivity of 58% and a specificity of 77% for diagnosing tuboperitoneal abnormalities (defined as at least unilateral tubal obstruction and/or hydrosalpinx and/or peri-adnexal adhesions) as compared to laparoscopy (Dabekausen *et al.*, 1994). A meta-analysis has been performed to determine the accuracy of HSG in diagnosing tubal patency and adhesions separately (Swart *et al.*, 1995). As compared to laparoscopy with tubal testing, HSG has a sensitivity of 65% and a specificity of 85% for diagnosing tubal patency, whereas HSG is unreliable for diagnosing peri-adnexal adhesions (Swart *et al.*, 1995). The low sensitivity of HSG (tubal pathology at laparoscopy despite normal HSG findings) may be due to peri-adnexal adhesions not visualized by the procedure or to incorrect interpretation of the HSG results. The specificity of HSG is higher, i.e. HSG is accurate in confirming tubal patency, but still approximately 25% of women with patent tubes at laparoscopy has abnormal HSG findings (Mol *et al.*, 1999). These false-positive HSG findings may be due to tubal spasms, dissimilar tubal filling pressure, too high viscosity of the contrast medium used or technical failure (Dabekausen *et al.*, 1994). Other disadvantages of HSG are that it is considered a painful test by patients and that it holds a risk of infection, which is up to 10% in patients with tubal pathology (Forsey *et al.*, 1990).
Because HSG has a limited predictive value for tubal disease and carries a risk of febrile morbidity, it has been questioned whether HSG is the best screening test in high-risk patients. Owing to the disadvantages of both laparoscopy and HSG, clinicians have tried to find an inexpensive and non-invasive test, which could accurately discern patients with a high versus low risk of tubal factor subfertility. Ideally, based on the results of such a screening test one would subject high-risk patients to diagnostic testing (i.e. laparoscopy), and delay additional invasive and expensive testing in low-risk patients. For this purpose, serological screening tests for tubal factor subfertility have been developed.

IgG to *C. trachomatis* (chlamydia IgG antibody testing)

Since the association between *C. trachomatis* immunoglobulin (Ig) G antibodies in serum and tubal pathology has been noted (Punnonen *et al.*, 1979), serum chlamydia IgG antibody testing (CAT) has been introduced as a screening test for tubal pathology in the fertility work-up. Following *C. trachomatis* infections, which mainly affect adolescents, a decade or more may pass until women present with subfertility. Serum IgG antibodies are known to remain detectable for many years (Gijsen *et al.*, 2002), even after antibiotic treatment (Puolakkainen *et al.*, 1986, Chaim *et al.*, 1992, Piura *et al.*, 1993, Henry-Suchet *et al.*, 1994). Therefore, in subfertile women CAT is considered a useful tool since it reflects a previous *C. trachomatis* infection. The costs of CAT are low (in the Netherlands about ten euros) (Fiddelers *et al.*, 2004) and the patient's discomfort is negligible.

The negative predictive value (NPV) of CAT in subfertile women is 85-90% (Mouton *et al.*, 2002, Veenemans and Van der Linden, 2002, Akande *et al.*, 2003, Land *et al.*, 2003, Logan *et al.*, 2003), although NPVs around 75% have been reported (Eggert-Kruse *et al.*, 1997, Tiitinen *et al.*, 2006). Because of the high NPV, the presence of tubal pathology in patients with a negative CAT is unlikely.
The positive predictive value (PPV) of CAT in subfertile women is lower than the NPV and ranges between 30% and 65% (Eggert-Kruse *et al.*, 1997, Mouton *et al.*, 2002, Veenemans and Van der Linden, 2002, Akande *et al.*, 2003, Land *et al.*, 2003, Logan *et al.*, 2003). The results reported on the diagnostic accuracy of CAT are heterogeneous due to differences in CAT tests, cut-off levels for a positive test, reference standard and definition of tubal pathology used (Land *et al.*, 1998, Land *et al.*, 2003) (Table III). However, the main limitation of CAT is the number of false-positive results, i.e. positive CAT in absence of tubal pathology, as reflected by the low PPV. A major concern of this high false-positive rate is that laparoscopies will be performed in women without tubal pathology. Unintended cross-reactivity with highly prevalent *C. pneumoniae* IgG antibodies has been suggested to account for false-positive test results in some CAT tests (Gijsen *et al.*, 2001, Land *et al.*, 2003). An even more important cause of false-positive CAT results is that a positive CAT is a marker of a previous *C. trachomatis* infection, but does not reflect the course of the infection and neither the eventual extent of the resulting tubal damage. CAT is incapable in discriminating between clearance and persistence of a *C. trachomatis* infection, while persistence is a major risk factor for tubal pathology. In order to screen for persistent *C. trachomatis* infections, the value of serological evidence of persistent *C. trachomatis* genital tract infections has been evaluated over the last few years in order to identify subfertile women at highest risk of tubal disease. In the following section, different serological markers of persistence will be described and discussed.

SEROLOGICAL MARKERS OF PERSISTENCE

IgG to chlamydia heat shock protein 60

Heat shock proteins (HSPs) are a family of proteins which are present in both *C. trachomatis* and humans. Their expression is up-regulated in response to stress, in order to protect the organism against stress-related damage. Chlamydia heat shock protein 60 (cHSP60) is a chlamydia genus-specific protein, serving as a strong antigenic target for the immune system (Morrison *et al.*, 1989, Kaufmann *et al.*, 1990). Antibodies to cHSP60 have been suggested as markers of chronic inflammation (Kaufmann *et al.*, 1990) and may therefore be good predictors for the risk of tubal pathology. Studies have shown a strong association between anti-cHSP60 antibod-

ies and tubal factor subfertility. Anti-cHSP60 antibodies are significantly more prevalent in subfertile women with tubal disease (44-79%) as compared to those without tubal disease or fertile controls (8-23%) (Freidank *et al.*, 1995, Claman *et al.*, 1997, Ault *et al.*, 1998, Persson *et al.*, 1999, Tiitinen *et al.*, 2006). Among subfertile women with antibodies to *C. trachomatis*, anti-cHSP60 antibodies are significantly more prevalent in women with tubal pathology (76-81%) as compared to those without tubal pathology (0-22%) (Toye *et al.*, 1993, Arno *et al.*, 1995). Heterogeneity between the results of the different studies may be due to methodologic differences, such as the type of cHSP60 IgG tests, cut-off levels, reference standard and definition of tubal pathology used. In particular, cross-reaction with the highly prevalent and highly similar *C. pneumoniae* HSP60 IgG may account for false-positive results. As predictors of tubal factor subfertility, cHSP60 IgG antibodies perform well, though not always superior to CAT (Persson *et al.*, 1999, Tiitinen *et al.*, 2006). It remains to be established whether cHSP60 IgG testing should be implemented in the fertility work-up as a screening method for *C. trachomatis*-associated tubal pathology.

Table III. Predictive value of different tests and cut-off levels for tubal pathology (TP) in subfertile women (adapted from Land *et al.*, 2003).

Chlamydia antibody test	Cut-off	No. of patients with positive test	No. of patients with positive test and TP	Sens (%)	Spec (%)	PPV (%)	NPV (%)	OR	95% CI
MIF Biomerieux	8	231	45	88	30	19	93	3.1	1.2-9.9
	16	149	39	76	58	26	93	4.6	2.1-10.3
	32	132	37	73	64	28	92	4.7[a]	2.3-10.2
	64	104	36	71	74	35	93	6.9	3.3-14.9
	128	60	29	57	88	48	91	9.9	4.7-21.1
MIF AniLabsystems	8	91	32	61	77	34	91	5.3	2.6-10.8
	16	75	32	61	83	41	92	7.8	3.7-16.2
	32	52	30	59	92	58	92	15.7[b]	7.1-35.1
	64	37	24	47	95	65	90	17.2	7.1-42.4
	128	19	13	25	98	68	87	14.7	4.6-52.4
ELISA AniLabsystems	Equivocal	84	23	45	77	27	88	2.7	1.3-5.4
	Positive	53	19	37	87	36	88	4.0[c]	1.9-8.4
	Highly positive	26	12	24	95	46	87	5.5	2.0-14.4
pELISA Medac	Equivocal	74	28	55	83	38	90	5.8	2.8-11.8
	Positive	62	28	55	87	45	91	8.2[d]	3.9-17.3
ELISA Savyon	Equivocal	99	26	51	72	26	88	2.7	1.4-5.4
	Positive	87	25	49	77	29	89	3.1[e]	1.6-6.3

[b] versus [a, c, d, e] and [d] versus [c, e] $P < 0.05$.

Abbreviations: CI = confidence interval; ELISA = enzyme-linked immunosorbent assay; MIF = micro-immunofluorescence; NPV = negative predictive value; OR = odds ratio; PPV = positive predictive value; sens = sensitivity; spec = specificity.

IgA to *C. trachomatis* and cHSP60

The presence of IgA antibodies has been proposed to reflect persistent infection more accurately as compared to IgG antibodies (Romano Carratelli *et al.*, 2006). Previous studies have demonstrated an association between *C. pneumoniae* IgA antibodies and its chronic sequelae, e.g. respiratory and cardiovascular morbidity (Saikku, 1999, Falck *et al.*, 2002, Wong *et al.*, 2002, Yavuz *et al.*, 2006). However, validated studies on the correlation between *C. pneumoniae* IgA antibodies and disease status are lacking and IgA antibody testing is therefore not recommended as a serological marker of persistent *C. pneumoniae* infection (Dowell *et al.*, 2001).
Contradictory findings have been reported on the value of *C. trachomatis* IgA antibodies in screening for tubal factor subfertility. Mouton and co-workers (2002) have found that IgA antibodies are more useful than IgG antibodies in diagnosing tubal pathology, whereas Paukku and co-workers (1998) have reported that IgG antibodies are better predictors for tubal pathology than IgA antibodies. These apparently contradictory findings may be due to methodological differences. Although the presence of serum IgA antibodies has been associated with chronic inflammation, the diagnostic accuracy is not superior to CAT or cHSP60 IgG testing. Therefore, *C. trachomatis* IgA antibody testing should not replace CAT in the fertility work-up.
The value of measuring cHSP60 IgA antibodies in subfertile women has been examined. CHSP60 IgA antibodies have been shown to be significantly more prevalent in subfertile women (regardless of the cause of subfertility) as compared to fertile controls (Karinen *et al.*, 2004). Dieterle and Wollenhaupt (1996) have found that cHSP60 IgA antibodies are significantly more prevalent in subfertile women with tubal pathology as compared to subfertile controls with patent tubes. So far, it is unclear whether cHSP60 IgA antibody testing is superior to CAT and whether it should deserve a role in screening for tubal factor subfertility.

IgG to *C. trachomatis* lipopolysaccharide

Lipopolysaccharides (LPS) are genus-specific components of the chlamydial envelope, which are acting as antigenic triggers for the immune system (Raulston *et al.*, 1995). Previous studies have suggested that anti-LPS antibodies are indicators of ongoing chlamydia infections (Tuuminen *et al*., 2000, Schumacher *et al.*, 2005, Kaklikkaya *et al.*, 2006). The prevalence of anti-LPS IgG antibodies has been found to be higher in patients with tubal factor subfertility as compared to fertile controls (Paukku *et al.*, 1998, Persson *et al.*, 1999). However, CAT and antibodies to cHSP60 have appeared to be better predictors of tubal factor subfertility (Persson *et al.*, 1999), indicating that LPS antibody testing has no additional value in the fertility work-up.

High-sensitivity C-reactive protein

The acute phase protein C-reactive protein (CRP) is a general serological marker of inflammation. CRP levels are > 10 mg/L in acute infections and < 1 mg/L in absence of an infection. CRP levels between 1 mg/L and 10 mg/L (so-called elevated levels within the normal range) are assumed to reflect a low-grade inflammation (Pearson *et al.*, 2003) and can be detected using a high-sensitivity (hs) CRP test. The role of elevated hs-CRP levels as markers of an ongoing low-grade inflammation has been evaluated in studies on the relationship between persistent *C. pneumoniae* infections and cardiovascular diseases. These studies have shown that the association between *C. pneumoniae* and cardiovascular diseases is even stronger in presence of slightly elevated hs-CRP levels (Roivainen *et al.*, 2000, Gattone *et al.*, 2001, Johnston *et al.*, 2001, Romano Carratelli *et al.*, 2006, Tiirola *et al.*, 2006, Yavuz *et al.*, 2006). So far, the value of hs-CRP in predicting the risk of fertility disorders following *C. trachomatis* genital tract infection has not been studied.

INTER-PATIENT VARIABILITY IN COURSE AND OUTCOME

A large inter-patient variability exists in the course and outcome of a *C. trachomatis* infection. Some women clear the infection adequately without developing tissue damage, whereas others develop a persistent infection, which increases the risk of tubal damage and may result in tubal factor subfertility. There is no generally accepted definition of persistence. From a clinical point of view, persistence involves exposure of the host to the pathogen during a longer period, increasing the risk of ascendance to the upper genital tract, endosalpingeal tissue damage and tubal factor subfertility. However, no consensus exists on the length of this period. From a scientific point of view, persistence is assumed to be characterized by a chronic low-grade immune response and/or the presence of aberrant *C. trachomatis* particles (AbdelRahman and Belland, 2005, Mpiga and Ravaoarinoro, 2006). In this thesis, we use both the clinical and scientific description of persistence. The course and outcome of infectious diseases are generally determined by virulence factors of the pathogen, environmental factors and host immune factors, and these factors will be discussed below.

Virulence factors of the pathogen

Several studies have evaluated whether different serovars are associated with differences in the clinical course of *C. trachomatis* infections, i.e. symptomatic versus asymptomatic infection, lower versus upper genital tract infection and clearance

versus persistence (Ito *et al.*, 1990, Persson and Osser, 1993, Dean *et al.*, 2000, Morré *et al.*, 2000, Geisler *et al.*, 2003, Molano *et al.*, 2005).
Serovars D, E and F account for the majority of *C. trachomatis* infections (Persson and Osser, 1993, Morré *et al.*, 2000, Geisler *et al.*, 2003, Molano *et al.*, 2005). Two studies reported a significant relationship between serovars and symptoms: serovar F (Geisler *et al.*, 2003) and the less-common serovar K (Morré *et al.*, 2000) were associated with a symptomatic course, whereas serovar Ia was found in asymptomatic women only (Morré *et al.*, 2000). However, both studies could not confirm each other's findings (Morré *et al.*, 2000, Geisler *et al.*, 2003), and Persson and Osser (1993) could not establish any relationship between serovars and symptoms.
In asymptomatic untreated patients, spontaneous clearance from the cervix occurred more often in women infected with the common serovars F and G, whereas persistent *C. trachomatis* infections were observed more frequently among serovars D and E and the less-common serovars B, H, I, J and K (Molano *et al.*, 2005). Remarkably, despite antibiotic treatment, serovars H, I and J were able to persist for two or three years in the lower genital tract of women (Dean *et al.*, 2000). In a mouse model, the duration of lower genital tract infection was longest with serovars D and E, and ascendance to the upper genital tract occurred more often in mice infected with serovar D as compared to mice infected with serovar H (Ito *et al.*, 1990).
Studies on the association between different *C. trachomatis* serovars and clinical course and outcome of the disease are not only relevant in the field of subfertility, but also in the field of oncology. It has been shown that *C. trachomatis* cervical infections are associated with cervical squamous cell carcinoma, but not cervical adenocarcinoma, by increasing the risk of persistence of the high-risk types of the oncogenic human papillomavirus (Samoff *et al.*, 2005, Quint *et al.*, 2009). Serovar studies have revealed that exposure to certain single *C. trachomatis* serovars (G, I and D) or to multiple *C. trachomatis* serovars is associated with the development of cervical squamous cell carcinoma (Anttila *et al.*, 2001).
In brief, studies on the association between virulence of the most common serovars and the course of *C. trachomatis* infections did not yield consistent and clinically applicable results. A hypothesis which is currently under investigation is that genetic variations in the plasticity zone (i.e. a virulence region in the bacterial genome) may account for intra-serovar or intra-strain differences in the course and outcome of *C. trachomatis* infections (Read *et al.*, 2000, Read *et al.*, 2003, Carlson *et al.*, 2004, Nelson *et al.*, 2006). Furthermore, a cryptic 7.5-kb plasmid has been shown to initiate and sustain *C. trachomatis* infection in the female mouse genital tract by regulating gene expression, and is therefore also proposed as a virulence factor (Carlson *et al.*, 2008).

Environmental factors

As mentioned earlier, the risk of tubal pathology is dependent on the number of episodes and the severity of PID (Weström, 1980, Weström *et al.*, 1992) (Table II). Furthermore, it has been suggested that the presence of multiple micro-organisms in the genital tract increases the risk of PID (Paisarntantiwong *et al.*, 1995), although little data are available. In large community-based and school-based screening programmes in the United Kingdom and in the United States of America, 4-12% of all *C. trachomatis*-infected women had a co-infection with other micro-organisms, such as *Neisseria* (*N.*) *gonorrhoeae* (Harindra *et al.*, 2002, Kent *et al.*, 2002, Miller *et al.*, 2004, Nsuami *et al.*, 2004). Studies in women attending clinics for sexually transmitted diseases (STDs) have shown a 13-28% rate of co-infections in *C. trachomatis*-infected women (Harindra *et al.*, 2002, Creighton *et al.*, 2003). *N. gonorrhoeae* infections are more often symptomatic than *C. trachomatis* infections. However, Nsuami and co-workers (2004) have reported that only 14% of women with both *C. trachomatis* and *N. gonorrhoeae* infections reported symptoms. This indicates that in the majority of women genital tract infections with these two micro-organisms will remain unnoticed and untreated, thereby increasing the risk of late sequelae.

Host immune factors

Because environmental factors and virulence factors do not seem to play a prominent role in the difference of the clinical course and outcome of *C. trachomatis* female genital tract infections, host immune factors are considered of more importance. Since especially pattern recognition receptors (PRRs) of the innate immune system are suggested to be involved in clearance of the infection, genetic variations in PRRs may contribute to an aberrant immune response and persistence, thereby increasing the risk of tubal pathology. In order to understand the contribution of host immune factors as determinants of the course and outcome of *C. trachomatis* infections, the normal immune response and its variations will be discussed.

THE NORMAL IMMUNE RESPONSE TO INFECTIONS

Innate immune system

The innate immune system is a general and non-specific system, which is the first line of defence against pathogens that are unknown to the host. Key elements of the innate immune system are macrophages, neutrophils, dendritic cells and natural killer cells. Several studies have suggested that besides the above-mentioned immune cells, epithelial cells play an important role in the early immune response to

infections (Rasmussen *et al.*, 1997, Quayle, 2002, Stephens, 2003, Mackern-Oberti *et al.*, 2006).

Both epithelial cells and circulating cells of the innate immune system possess cell-surface-bound or intracellular PRRs. The two most important families of PRRs are the toll-like receptor (TLR) family and the nucleotide-binding oligomerization domain (NOD) proteins. PRRs recognize and bind pathogen-associated molecular patterns (PAMPs), which are components on and in foreign organisms. Binding of a PRR to its PAMP initiates several intracellular reactions, including a signal transduction cascade with nuclear factor (NF)-κB as the end product. NF-κB is able to bind to specific DNA sequences in the nucleus, thereby enhancing the production of (amongst others) pro-inflammatory cytokines. Some PRRs, such as cluster of differentiation (CD) 14, (partly) exist in a soluble extracellular form and act as a co-receptor. Initiation of the immune response then occurs by binding of an extracellular PAMP-PRR complex to a transmembrane PRR. Because different PRRs recognize different PAMPs, the PRR system provides a complex and flexible initiation of the innate immune response. Figure II shows the initiation of the innate immune response by PAMP-PRR complexes.

When a pathogen enters the body, epithelial cells are the first line of defence. The epithelial PRRs bind to the pathogen, and the epithelial cells start to secrete chemokines (which attract circulating cells of the innate immune system to the site of infection) and other pro-inflammatory cytokines. When the circulating cells of the innate immune system arrive at the site of infection, their PRRs bind to the pathogen. Subsequently, macrophages, neutrophils and dendritic cells ingest the pathogen by phagocytosis and destroy it within the cell. Natural killer cells directly destroy the pathogen by cytolysis. Macrophages and dendritic cells are able to express pathogen-components (antigens) bound to major histocompatibility complex-proteins (also known as human leucocyte antigens) on their surface and to serve as antigen-presenting cells (APCs), which can activate the acquired immune system. Circulating cells of the innate immune system also produce pro-inflammatory cytokines.

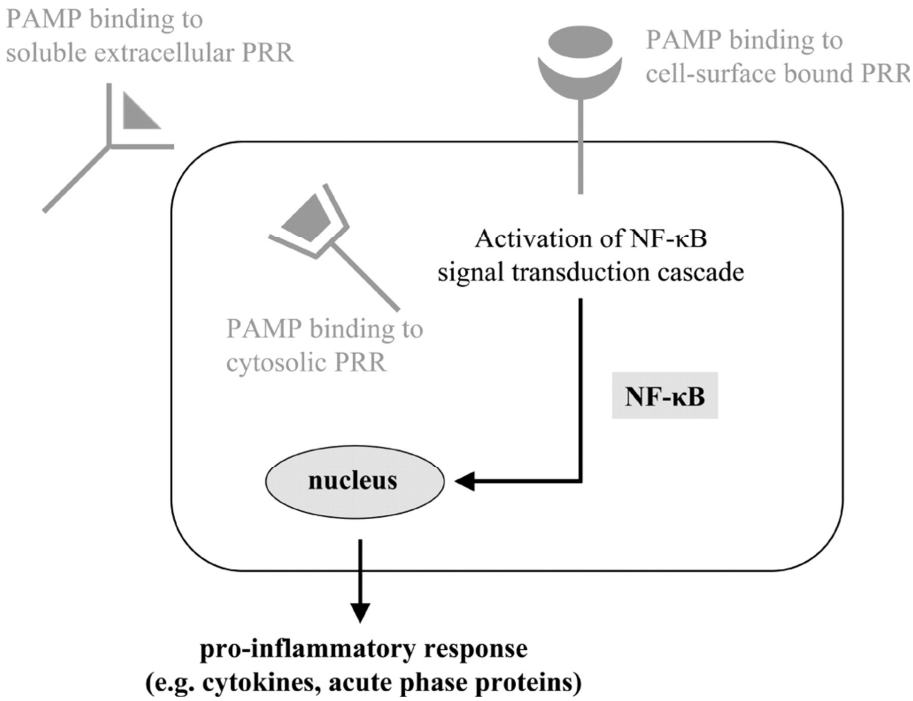

Figure II. The initiation of the innate immune response.
The initiation of the innate immune response starts by binding of pathogen-associated molecular patterns (PAMPs) to their pattern recognition receptors (PRRs) on or in circulating cells of the innate immune system (e.g. macrophages, neutrophils, dendritic cells, natural killer cells) or local epithelial cells or to soluble extracellular PRRs. This leads to activation of the nuclear factor (NF)-κB signal transduction cascade. Its end product NF-κB binds to specific DNA sequences in the nucleus, thereby enhancing the production of pro-inflammatory cytokines and acute phase proteins.

Acquired immune system

The acquired (or adaptive) immune system is a specific system, which develops after the first contact with a pathogen. It builds up a memory against the pathogen, which is responsible for a quick immune response following re-infection. The acquired immune system consists of a humoral arm (with B lymphocytes, mainly targeting extracellular pathogens) and a cell-mediated arm (with T lymphocytes, mainly targeting intracellular pathogens), which closely interact.
In the humoral arm, B lymphocytes are activated by APCs (cells of the innate immune system or T lymphocytes). Activated B lymphocytes develop into plasma cells and produce antibodies which neutralize the antigen or directly destroy the pathogen. An antibody-antigen complex can also activate the complement system. Furthermore, B lymphocytes can serve as APCs for T lymphocytes.

In the cell-mediated arm, T lymphocytes are activated by APCs (cells of the innate immune system or B lymphocytes). Most T lymphocytes are T helper (Th) cells. Th cells produce pro-inflammatory cytokines. The Th1 subclass produces interleukin (IL)-12 and interferon γ, which support the cell-mediated system. The Th2 subclass produces IL-4, IL-5, IL-6 and IL-10, which support the humoral system. The relative contributions of the two respective subclasses of Th cells determine whether the cell-mediated or the humoral arm is predominant. Cytotoxic T cells (or killer cells) directly attack and destroy a pathogen and produce pro-inflammatory cytokines. Suppressor T cells provide a negative feedback mechanism to protect the host against an excessive immune response (i.e. hyperinflammation).

Complement system

The complement system consists of a group of over twenty proteins. Most of them are circulating in an inactive form (precursors). Once the complement system is activated, a cascade of reactions leads to active end products, which enhance the immune response or destroy the pathogen. Activation of the complement system can be induced by an antibody-antigen complex (classical pathway) or by membrane components of the pathogen (alternate pathway).

RECOGNITION OF *C. TRACHOMATIS*

Clearance of a *C. trachomatis* infection

In most women a normal immune response to a *C. trachomatis* infection will occur, resulting in adequate clearance (reviewed by Golden *et al.*, 2000, Joyner *et al.*, 2002, Morré *et al.*, 2002, Molano *et al.*, 2005, Geisler *et al.*, 2008). The host is exposed to the pathogen during a short period of time, leading to no or minimal tissue damage. A key element of a normal immune response to a *C. trachomatis* infection is an adequate recognition of the pathogen by PRRs on and in epithelial cells in the genital tract and the initiation of the immune response. The role of PRRs of the TLR and NOD families in *C. trachomatis* recognition and an early initiation of the immune response will be discussed (summarized in Table IV).

TLRs

TLRs are cell-surface-bound or intracellular PRRs. So far, eleven different TLRs have been identified. The PAMPs of all TLRs, except TLR10, are known (Akira and Takeda, 2004). Binding of a TLR to its PAMP initiates the immune response by triggering the NF-κB signal transduction cascade. It is plausible that TLRs play a role in the host

defence mechanism against *C. trachomatis* genital tract infections, because some TLRs are able to recognize *C. trachomatis* PAMPs, and are expressed in epithelial cells in the human genital tract.

Table IV. Presence of TLRs and NODs in the genital tract and their common genetic variations.

PRR	PAMP	Presence in genital tract				Common genetic variation
		Human studies		Animal studies		
		In vivo	*In vitro*	*In vivo*	*In vitro*	
TLR2	Peptidoglycan	+	+	+	+	GT repeat in intron 2 Arg753Gln -16933 T>A
TLR4	LPS and HSP	+	+	+	+/-	+896 A>G (Asp299Gly) +1196 Thr399Ile
TLR9	Bacterial DNA	NA	+	NA	NA	-1237 T>C +2848 G>A
NOD1	Peptidoglycan	NA	NA	NA	+	Del T/Ins GG +32656
NOD2	Peptidoglycan	NA	NA	NA	+	2023 C>T (SNP8, R675W) 2641 G>C (SNP12, G1881R) 2936insC (SNP13, Leu1007fsinsC, 980fs981X)

+ = Present in genital tract. +/- = Present in genital tract in some, but not all, studies.
Abbreviations: HSP = heat shock protein; LPS = lipopolysaccharide; NA = not analyzed; NOD = nucleotide oligomerization domain; PAMP = pathogen-associated molecular pattern; PRR = pattern recognition receptor; TLR = toll-like receptor.

TLR2 is the PRR for the *C. trachomatis* component peptidoglycan (Schwandner *et al.*, 1999, Yoshimura *et al.*, 1999), and TLR4 is the PRR for the *C. trachomatis* components LPS and HSP (Poltorak *et al.*, 1998, Ohashi *et al.*, 2000). TLR2 and TLR4 are expressed in the human female genital tract (Pioli *et al.*, 2004, Fazeli *et al.*, 2005) and in epithelial cell lines derived from the human genital tract (Schaefer *et al.*, 2004, O'Connell *et al.*, 2006). TLR2 is also expressed in a cloned murine tubal epithelial cell line (Derbigny *et al.*, 2005). Differential expression along the human genital tract has been observed for TLR2, mainly expressed in the tubes and cervix, and for TLR4, mainly expressed in the tubes and endometrium and weakly expressed or even absent in the ectocervix (Pioli *et al.*, 2004, Fazeli *et al.*, 2005). These differences in expression may be related to the different functions of the different parts of the genital tract: protection against sexually transmitted pathogens without disturbing the functional vaginal commensal flora and toleration of semen and embryonic implantation. Up-regulation of TLR2 and TLR4 expression following chlamydial infection has been noted in several studies (Mackern-Oberti *et al*., 2006, O'Connell *et al.*, 2006).

TLR9 recognizes bacterial DNA (Hemmi *et al.*, 2000). So far, the expression of TLR9 has not been studied in the human female genital tract, although TLR9 expression has been found in the human uterine epithelial cell line ECC-1 (Schaefer *et al.*,

2004). Its precise role in *C. trachomatis* female genital tract infections remains to be established.
TLR1, TLR3, TLR5 and TLR6 are also present in the human female genital tract (Pioli *et al.*, 2004, Fazeli *et al.*, 2005), but they do not recognize *C. trachomatis* PAMPs. This suggests that these TLRs may play a role in the host defence against non-*C. trachomatis* and/or polymicrobial genital tract infections.
Animal studies are able to provide information on the role of PRRs in *C. trachomatis* infections that cannot be obtained by human studies, although results of animal studies may not be freely translated to the human *in vivo* situation and the strain of *C. trachomatis* used (e.g. *C. muridarum* versus serovars D-K versus the more invasive LGV serovar) may influence the outcome in any of the models used. Knockout (KO) mouse technology offers the opportunity to remove entire genes of interest from the genome and to compare the course and outcome of infectious diseases between KO mice and wild-type (WT) mice, which possess the gene of interest. Darville and co-workers (2003) have designed a KO mouse model to study the role of TLR2 and TLR4 in the course and outcome of a *C. muridarum* infection, which is the mouse variant of *C. trachomatis*. WT mice with normal TLR2 and TLR4 genes served as controls. The *in vitro* cytokine production of macrophages was down-regulated, but not totally inhibited, in macrophages derived from TLR2 KO mice, whereas it was up-regulated in macrophages derived from TLR4 KO mice. The *in vivo* resolution of a *C. muridarum* infection was equally efficient in KO and WT mice, indicating that the remaining and/or compensatory immune mechanisms seem to lead to sufficient clearance in KO mice. Remarkably, TLR2 KO mice developed less tubal pathology in comparison with WT mice, despite a down-regulated cytokine production. These findings suggest that TLR2 genetic variations may provide a balanced immune response leading to efficient clearance, rather than hypo- or hyperinflammation, and serve as protection against tissue damage (Darville *et al.*, 2003).

NODs

NOD proteins are intracellular PRRs. The family of NOD proteins contains at least 25 proteins, including NOD1 and NOD2 (Inohara and Nuňez, 2003). NOD1 and NOD2 are also referred to as caspase recruitment domain (CARD) 4 and CARD15, respectively. NODs are able to recognize intracytoplasmatic bacterial PAMPs, such as LPS and peptidoglycan (Inohara *et al.*, 2001, Girardin *et al.*, 2003). Binding of a NOD to its PAMP activates the NF-κB signal transduction cascade, which initiates the immune response.
Because *C. trachomatis* is an intracellular pathogen containing LPS and peptidoglycan, a role of intracellular NODs in the recognition of *C. trachomatis* has been suggested. This is supported by findings of several studies (Derbigny *et al.*, 2005, Opitz *et al.*, 2005, Welter-Stahl *et al.*, 2006, Buchholz and Stephens, 2008). Welter-Stahl

and co-workers (2006) have found that *C. trachomatis* produces at least the rudimentary proteoglycan motif recognized by NOD1. Furthermore, it has been proven that NOD1 and NOD2 are expressed in a cloned murine fallopian tube epithelial cell line (Derbigny *et al.*, 2005). *C. pneumoniae* has been shown to induce a NOD-mediated pro-inflammatory immune response in endothelial cells *in vitro* (Opitz *et al.*, 2005). The most recent study has concluded that, in epithelial cells *in vitro*, the inflammatory response following *C. trachomatis* infection is NOD1-dependent (Buchholz and Stephens, 2008).

PERSISTENCE OF A *C. TRACHOMATIS* INFECTION

In some women, a *C. trachomatis* infection will not be cleared adequately and may result in a persistent infection. The host is exposed to the pathogen during a longer period of time, increasing the risk of ascendance to the upper genital tract, endosalpingeal tissue damage and tubal factor subfertility. The course of a *C. trachomatis* infection (i.e. whether the infection will be cleared or persist) may be determined by host immune factors.

Introduction to immunogenetics

Although differences in the infectivity among *C. trachomatis* strains have been reported and reinfection has been shown to influence the clinical course, at this time neither virulence factors of the pathogen nor environmental factors do seem to play a major role in the difference of the clinical course of *C. trachomatis* infections, suggesting that host immune factors are to be considered more important determinants of the inter-patient variability in the course and outcome.
Immunogenetic studies evaluate the role of genetic variations in immunologically important host genes in the course and outcome of infectious diseases. Among these genetic variations are single nucleotide polymorphisms (SNPs), in which one nucleotide has been substituted, inserted or deleted, and variations in the number of repetitive DNA sequences (variable number of tandem repeats). Carrying a genetic variation may have direct or indirect biological consequences. Potential direct biological consequences of carrying a genetic variation are translation of an aberrant protein, or up- or down-regulation of the translation of a normal protein. If a genetic variation is not functional, i.e. does not change the function of the gene studied, it may have indirect biological consequences when it is inherited together with another, sometimes still unidentified, functional gene nearby (linkage).
During the past years, immunogenetic studies have provided more insight in the inter-patient variability in the course and outcome of infectious diseases. Several studies have found an association between carriage of genetic variations and infec-

tious diseases, such as hepatitis, inflammatory bowel diseases, meningococcal infections and *Ureoplasma urealyticum* lower genital tract infections (Jeremias *et al.*, 1999, Smirnova *et al.*, 2003, Franchimont *et al.*, 2004, Peeters *et al.*, 2004, Frodsham, 2005). Regarding *C. trachomatis* ocular infections, a 40% genetic predisposition was noted in a Gambian twin study, supporting the relevance of genetics in *C. trachomatis* infections (Bailey *et al.*, 1998, Bailey *et al.*, 2009).
As discussed previously, a normal immune response to a *C. trachomatis* infection is based on an adequate recognition of the pathogen by, amongst others, TLRs and NODs on epithelial cells in the genital tract. In the next paragraphs, the role of genetic variations in genes encoding TLRs and NODs as potential risk factors for persistent *C. trachomatis* infections will be discussed (see also Table IV).

Variations in *TLR* genes

It is likely that TLR2, TLR4 and TLR9 play a role in the recognition of *C. trachomatis* in the genital tract, because they are expressed in the human female genital tract and because they are able to recognize *C. trachomatis* PAMPs. It is assumed that genetic variations in *TLR* genes may result in aberrant receptor density on or in cells or in dysfunctional receptors, leading to an inadequate recognition of *C. trachomatis* and an increased risk of persistence. However, not many human studies have tested this hypothesis.
Regarding TLR4, it is known that only homozygous carriage of the *TLR4* +896 A>G (also referred to as Asp299Gly) and Thr399Ile SNPs affects the LPS receptor function, whereas heterozygous carriage has no effect on the LPS receptor function (Erridge *et al.*, 2003). Homozygous carriage of the *TLR4* +896 A>G SNP is rare. In a study comparing 30 CAT-positive subfertile women with tubal pathology with 41 CAT-positive subfertile women without tubal pathology, only one woman carried the homozygous *TLR4* +896 A>G SNP, and no significant association between this SNP and *C. trachomatis*-associated tubal pathology could be found (Morré *et al.*, 2003). So far, the latter two studies are the only human studies on the role of *TLR* genetic variations and susceptibility to *C. trachomatis* genital tract infections. The number of human studies may be limited due to difficulties in collecting adequate sample sizes, because patients who have undergone a *C. trachomatis* infection ánd have had evaluation of the tubal function ánd carry a single or multiple genetic variations are exceedingly rare. Multi-center trials might resolve this drawback. Although functional SNPs in the *TLR2* gene have been described in relation to infection and inflammation (Lorenz *et al.*, 2000, Sutherland *et al.*, 2005, Yim *et al.*, 2006), no studies have yet been performed for *C. trachomatis* infections.
Further studies are needed to investigate the precise role of TLRs in *C. trachomatis* genital tract infections, in particular to determine whether *TLR* genetic variations

act in a damaging way, as generally assumed, or in a protective way, as suggested by Darville and co-workers in their KO mouse model (2003).

Variations in *NOD* genes

The precise role of NOD proteins in the intracellular recognition of *C. trachomatis* in the genital tract has not been established, although several studies suggest that NODs are involved in the immune response to *C. trachomatis* genital tract infections (Inohara *et al.*, 2001, Girardin *et al.*, 2003, Derbigny *et al.*, 2005, Opitz *et al.*, 2005, Welter-Stahl *et al.*, 2006, Buchholz and Stephens, 2008). If this association could be confirmed, *NOD* genetic variations may be risk factors of inadequate recognition and persistence in *C. trachomatis* infections.
Several genetic variations in the *NOD2* genes have been associated with the susceptibility to inflammatory bowel disease (Hugot *et al.*, 2001, Ogura *et al.*, 2001, Hampe *et al.*, 2002, Murillo *et al.*, 2002, McGovern *et al.*, 2005). Carrying a *NOD2* genetic variation seems to result in hyporesponsiveness to enteric bacteria, increasing the risk of chronic bowel inflammation. Hugot and co-workers (2001) have also identified a so-called gene-dosage effect: the higher the number of genetic variations in a patient, the higher the risk of Crohn's disease. As compared to patients without *NOD2* variations, the relative risk of Crohn's disease was 3 in heterozygous carriers of a single variation, 38 in homozygous carriers of a single variation and 44 in heterozygous carriers of two variations (Hugot *et al.*, 2001). In a recent study, this gene-dosage effect has also been demonstrated for *NOD2* variations and variations in other disease-associated genes in Crohn's disease (Weersma *et al.*, 2009).
As NOD2, NOD1 is also a ubiquitous cytosolic receptor for peptidoglycan from Gram-negative bacteria, and recent studies have suggested that *C. trachomatis* and *C. muridarum* do, in fact, produce at least the rudimentary proteoglycan motif recognized by NOD1. Welter-Stahl and co-workers (2006) have demonstrated that NOD1 deficiency has no effect on the duration of chlamydia infection, the intensity of cytokine secretion, or the extent of pathology in KO mice, compared with WT controls. Thus, chlamydia may not produce sufficient peptidoglycan to stimulate NOD1-dependent pathways efficiently, or other receptors of the innate immune system may compensate for the absence of NOD1 during chlamydia infection *in vivo*, as has been shown by Netea and co-workers (2005). But, on the contrary, a recent *in vitro* study has found reduced IL-8 levels in NOD1 knockdown HeLa cells following *C. trachomatis* infection, suggesting that the inflammatory response to *C. trachomatis* infection is NOD1 dependent (Buchholz and Stephens, 2008).
The studies mentioned encourage to investigate whether NODs play a role as PRRs for *C. trachomatis*, and if so, whether genetic variations increase the risk of an aberrant immune response and persistence of *C. trachomatis* infections.

Conclusive remarks on immunogenetics

Carriage of a single variation in a single host gene does not necessarily lead to an increased risk of developing late sequelae of infectious diseases, especially in case of a polygenic multivariate infection like *C. trachomatis*. The immune system is a complex and flexible system, and compensatory routes will, to a certain extent, provide alternative pathways to trigger the immune response. For instance, disfunctionality of the NOD1 pathway can be partially overcome by functional TLRs (Netea *et al.*, 2005), and not only PRRs but also the complement system is involved in pathogen recognition. Furthermore, heterozygous carriage of some genetic variations may not have a large effect on the function of the gene (Erridge *et al.*, 2003) because the other allele can compensate. It is also hypothesized that the risk of late sequelae increases with the number of genetic variations, as found for variations in NODs and other disease-associated genes in Crohn's disease (Hugot *et al.*, 2001, Weersma *et al.*, 2009) and for TLR variations in meningococcal infections (Smirnova *et al.*, 2003).
In general, the main goal of immunogenetic studies is to provide more insight in the immunopathogenesis of infectious diseases and to identify genetic markers related to the susceptibility to and severity of infection. The end goal is to identify patients at highest risk of complications and to assess whether immunogenetic analyses can be applied in screening for tubal pathology. Regarding *C. trachomatis* female genital tract infections, the precise role of PRRs and their genetic variations remains to be elucidated.

AIMS OF THE THESIS

The thesis focusses on persistent *C. trachomatis* infections and the ensuing tubal factor subfertility. The first aim of the thesis is to improve the predictive value of CAT in assessing the risk of *C. trachomatis*-associated tubal factor subfertility. It is hypothesized that serological markers of persistent *C. trachomatis* infections can be used to identify subfertile women at highest risk of tubal pathology. In addition, different screening strategies for tubal factor subfertility which can be applied in the fertility work-up are appraised. Secondly, the role of host immunogenetic factors contributing to the course of *C. trachomatis* infections is explored. Furthermore, different detection methods for persistent *C. trachomatis* in endometrial tissue of subfertile women are studied.

OUTLINE OF THE THESIS

The first part of the thesis describes different screening methods for the risk assessment of *C. trachomatis*-associated tubal factor subfertility. The risk of developing tubal pathology following *C. trachomatis* infection depends on the course of the infection. Most women will effectively clear a *C. trachomatis* infection and have a low risk of developing complications. However, women with a persistent *C. trachomatis* infection are assumed to be at highest risk for tubal factor subfertility. In **Chapter 2**, it will be evaluated whether serological markers of persistence (*C. trachomatis* IgA antibodies, cHSP60 IgG antibodies and hs-CRP) can identify women at highest risk of tubal factor subfertility. In **Chapter 3**, anti-LPS IgG antibodies will be tested as potential markers of persistence. Furthermore, the contribution of *C. pneumoniae* and *C. psittaci* infections, following a primary *C. trachomatis* infection, to the development of tubal pathology will be studied. In **Chapter 4**, three screening strategies to estimate the risk of tubal pathology in subfertile women will be explored. The screening strategies comprise *C. trachomatis* IgG antibody testing, hs-CRP and HSG. One of the screening strategies is based on our findings described in Chapter 2.

The second part of this thesis focusses on immunogenetic aspects of *C. trachomatis*-associated tubal factor subfertility. Amongst others, SNPs (i.e. genetic variations) in immunologically important host genes are determinants of the inter-patient variability of *C. trachomatis* infections. In **Chapter 5**, the *TLR4* +896 A>G SNP will be studied in relation to the susceptibility to *C. trachomatis* infections (in a cohort of women who visited an outpatient STD clinic) and outcome of *C. trachomatis* infections (in a cohort of subfertile women). Additionally, a knock-out mouse model will be used to test the role of TLR4 in the susceptibility and outcome of primary *C. trachomatis* infections and re-infections. In **Chapter 6**, the role of the *CD14* -260 C>T SNP in the susceptibility to and severity of *C. trachomatis* infections will be evaluated in the STD cohort and subfertility cohort. In **Chapter 7**, the hypothesis that carrying multiple SNPs in pattern recognition receptor genes, rather than a single SNP, may result in the highest risk of tubal pathology following *C. trachomatis* infection will be tested.

The third part of this thesis addresses the question whether endometrial sampling can be used as a diagnostic method for identifying persistent *C. trachomatis* upper genital tract infection in subfertile women. In **Chapter 8**, a pilot study using immunohistochemical staining methods and polymerase chain reaction will be performed to detect *C. trachomatis* in the endometrium of subfertile women. The results will be correlated to the presence of C*. trachomatis* IgG antibodies in serum and the presence of tubal pathology at laparoscopy.

In **Chapter 9**, the results will be summarized. Recommendations for clinical application and future research will be presented.

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Chapter 2

Serological markers of persistent *Chlamydia trachomatis* infections in women with tubal factor subfertility

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ABSTRACT

Background
Persistent *Chlamydia* (*C.*) *trachomatis* infections are assumed to increase the risk of tubal pathology. We studied whether serological markers, assumed to be associated with persistent *C. trachomatis* infections, could identify subfertile women at risk of tubal pathology.

Methods
Sera of 313 subfertile women, who all underwent a laparoscopy with tubal testing to assess the grade of tubal pathology, were tested for the presence of immunoglobulin (Ig) G and IgA antibodies to *C. trachomatis,* IgG antibodies to chlamydia heat shock protein 60 (cHSP60) and high-sensitivity C-reactive protein (hs-CRP).

Results
C. trachomatis IgA, cHSP60 IgG and hs-CRP, all serological markers of persistent infections, were significantly more prevalent in women with tubal pathology as compared to those without tubal pathology. The predictive value of the currently used screening test for tubal pathology (IgG to *C. trachomatis*) could be significantly improved by adding the hs-CRP test.

Conclusions
In subfertile women with tubal pathology, serological markers of persistent *C. trachomatis* infections are significantly more common as compared to women without tubal pathology. *C. trachomatis* IgG-positive subfertile women with slightly elevated (< 10 mg/L) hs-CRP levels are at highest risk of persistent *C. trachomatis* infections and tubal pathology.

INTRODUCTION

Chlamydia (*C.*) *trachomatis* infections are a major cause of tubal factor subfertility. However, the precise pathogenesis of *C. trachomatis* infections remains to be elucidated. Not all women who have undergone a *C. trachomatis* infection will develop tubal pathology. Host factors, virulence factors of the micro-organism and environmental factors determine the course and morbidity of *C. trachomatis* infections. Depending on these factors and their interaction, *C. trachomatis* infections will either be cleared or persist. A clearance rate of 44.7% has been reported in asymptomatic and untreated women after one year follow-up (Morré *et al.*, 2002). In women who clear a *C. trachomatis* infection adequately, the risk of tubal damage may be low, since the host has been exposed to the micro-organism during a short period. Persistent exposure to the micro-organism may result in a chronic inflammatory response and may increase the risk of tubal damage, as has been suggested previously (Grayston *et al.*, 1985, Patton *et al.*, 1994a).

Previous studies, in which evidence of persistent *C. trachomatis* infections has been found in the upper genital tract of women with tubal damage, support this hypothesis. Gérard *et al.* (1998) have found viable *C. trachomatis* micro-organisms in seven out of ten tubes of patients with ectopic pregnancies. Furthermore, *C. trachomatis* has been detected in 56–79% of the tubes of women with tubal factor subfertility, who underwent reconstructive tubal surgery (Campbell *et al.*, 1993, Patton *et al.*, 1994b). Previously, we have demonstrated that genus-specific immunoglobulin (Ig) G antibodies to chlamydia lipopolysaccharide, which are supposed to be markers of persistent infections, are significantly more often detectable in sera of subfertile women with distal tubal pathology (62.7%) as compared to those without distal tubal pathology (33.9%) (Den Hartog *et al.*, 2004).

Since the association between *C. trachomatis*-specific IgG antibodies and tubal pathology has been noted (Punnonen *et al.*, 1979), measuring *C. trachomatis* IgG antibodies in serum is used as a screening method for tubal pathology. Although species-specific *C. trachomatis* IgG antibodies are markers of previous infections, their presence does not reflect the course of the infection. Therefore, measuring IgG antibodies to *C. trachomatis* is not useful in discriminating between clearance or persistence of the infection.

We hypothesize that persistent *C. trachomatis* infections play an important role in the development of tubal pathology, and have studied known serological markers of persistent infections in subfertile women with and without tubal pathology. Elevated levels of IgA antibodies and C-reactive protein (CRP), in the absence of an acute infection, have been suggested to be markers of chronic inflammation and infection, and have been evaluated previously in studies on the relationship between chronic *C. pneumoniae* infections and respiratory and cardiovascular disease (Roivainen *et al.*, 2000, Gattone *et al.*, 2001, Johnston *et al.*, 2001, Falck *et al.*, 2002,

Wong *et al.*, 2002). IgG antibodies to chlamydia heat shock protein 60 (cHSP60) have also been associated with chronic inflammation (Morrison *et al.*, 1989), and have been studied previously in subfertile women with tubal pathology (Toye *et al.*, 1993, Arno *et al.*, 1995, Freidank *et al.*, 1995, Claman *et al.*, 1997).
Sera of subfertile women were tested for the presence of IgG and IgA antibodies to *C. trachomatis*, IgG antibodies to cHSP60 and CRP. All women underwent a laparoscopy with tubal testing. We correlated the serological test results with the presence of tubal pathology at laparoscopy, and evaluated the role of single tests and test combinations in predicting the risk of persistent *C. trachomatis* infections and tubal pathology.

MATERIALS AND METHODS

The study was performed in subfertile women who entered our clinic between December 1990 and November 2000. As part of their fertility work-up, in all female patients blood was drawn at their initial visit for a chlamydia IgG antibody test (CAT). All spare sera were cryopreserved and thawed for this study. Patients with a negative CAT and an otherwise normal fertility work-up underwent a hysterosalpingography (HSG) to evaluate the tubal status. If the HSG showed abnormalities, or if they did not conceive within 6 months after the HSG, a laparoscopy with tubal testing was performed. Patients with a positive CAT underwent a laparoscopy with tubal testing immediately after the fertility work-up. Only patients who had undergone a laparoscopy and tubal testing with methylene blue dye were included in the present study. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded.
Two independent investigators, who were unaware of the CAT results, scored 313 successive laparoscopy reports to assess the grade of tubal pathology. In cases of disagreement, consensus was reached by consultation. For the sake of the study, tubal pathology was defined as extensive peri-adnexal adhesions and/or distal occlusion of at least one tube (Land *et al.*, 1998). Subfertile women without distal tubal pathology served as controls. The controls had unexplained subfertility, partners with mild male factor subfertility, or proximal occlusion of at least one tube.

Serological methods

IgG antibodies to *C. trachomatis* were detected using the species-specific *Chlamydia pneumoniae* IgG micro-immunofluorescence (MIF) test (AniLabsystems, Finland), as described previously (Den Hartog *et al.*, 2004). This species-specific test, which is the currently used screening test for *C. trachomatis* IgG antibodies in our clinic, has been

found to be a good predictor of tubal pathology (Land *et al.*, 2003). The threshold titre used for a positive test was 32.
IgA antibodies to *C. trachomatis* were detected using the *Chlamydia trachomatis* IgA enzyme immunoassay (AniLabsystems, Finland). The test was used according to the manufacturer's instructions. The threshold index for a positive test was 1.4.
IgG antibodies to cHSP60 were detected using the cHSP60 IgG enzyme-linked immunosorbent assay (ELISA; Medac, Germany), which is available for research use only. The test was used according to the manufacturer's instructions. The threshold index for a positive test was 1.11.
CRP was determined using the CRP ELISA (DiaMed Eurogen, Belgium). The test was used according to the manufacturer's instructions. In order to reliably detect low CRP concentrations, this high-sensitivity (hs) CRP test was used. CRP levels between 1.0 and 10.0 mg/L (slightly raised levels, but still within the normal range) are assumed to reflect a persistent infection, and were considered as positive. CRP levels < 1.0 mg/L (low risk of persistent infection) or > 10.0 mg/L (probably acute infection) were considered as negative (Pearson *et al.*, 2003).

Statistical methods

Characteristics of women with and without distal tubal pathology were compared using the Mann–Whitney *U*-test. For comparison of the prevalence of IgG and IgA antibodies to *C. trachomatis*, IgG antibodies to cHSP60 and hs-CRP in women with and without distal tubal pathology, the χ^2-test was used. The prognostic value of single tests as well as test combinations for distal tubal pathology was determined by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), odds ratio (OR) and 95% confidence interval (CI). A forward stepwise logistic regression analysis was used to select the best combination of tests. The bootstrap technique was used to test the difference between ORs (Efron and Tibshiram, 1993). $P < 0.05$ was considered statistically significant.

RESULTS

In 313 subfertile women, serological test results and laparoscopy reports were available for analysis. Of those 313 women, 59 (18.8%) met the definition of distal tubal pathology (extensive peri-adnexal adhesions and/or distal occlusion of at least one tube), whereas 254 women (81.2%) did not have distal tubal pathology and served as controls. Of those 254 women without distal tubal pathology, 94.9% had patent tubes and 5.1% had proximal occlusion of at least one tube. Since proximal tubal occlusion is considered not to be related to chlamydia disease, all 254 women without distal tubal pathology served as controls. In women with and without distal

tubal pathology, mean age (30.6 and 31.2 years, respectively) and duration of subfertility (2.4 and 2.3 years, respectively) were comparable.

CRP levels between 1.0 and 10.0 mg/L (slightly raised levels, but still within the normal range) were assumed to reflect a persistent infection, and were considered as positive. CRP values > 10.0 mg/L were considered as negative, since these values were assumed to reflect acute infections. Twelve women (3.8%) had CRP levels > 10.0 mg/L (median 16.1, range 11.2—> 130.3). Of these twelve patients, two had severe endometriosis, which is a known cause of elevated CRP levels (Abrão *et al.*, 1997). In the remaining ten patients, no clinical evidence of acute infections or other underlying inflammatory diseases could be found. Since seven out of these ten serum samples were obtained in autumn or winter, minor or subclinical infections (e.g. influenza-like infections) might have caused the elevated CRP levels.

First, we evaluated the prevalence of IgG and IgA antibodies to *C. trachomatis*, IgG antibodies to cHSP60 and a positive hs-CRP in subfertile women with and without distal tubal pathology. As shown in Table I, IgG and IgA antibodies to *C. trachomatis*, IgG antibodies to cHSP60 and a positive hs-CRP test were found significantly more often in women with distal tubal pathology as compared to women without distal tubal pathology.

Table I. The prevalence of positive tests in subfertile women with and without distal tubal pathology.

	DTP (*n*=59)	No DTP (*n*=254)	*P*
Ctr-IgG +[a]	32 (54.2)	20 (7.9)	< 0.05
Ctr-IgA +[b]	21 (35.6)	21 (8.3)	< 0.05
cHSP60-IgG +[c]	30 (50.8)	38 (15.0)	< 0.05
hs-CRP +[d]	32 (54.2)	95 (37.4)	< 0.05

Values in parentheses are percentages; [a] Threshold titre for a positive test: 32.; [b] Threshold index for a positive test: 1.4; [c] Threshold index for a positive test: 1.11; [d] Threshold concentration for a positive test: 1.0–10.0 mg/L. Abbreviations: cHSP60=chlamydia heat shock protein 60; Ctr= *C. trachomatis*; DTP = distal tubal pathology; hs-CRP= high-sensitivity C-reactive protein; Ig = immunoglobulin.

Table II shows that, of all four single tests, the *C. trachomatis* IgG test was the best predictor of tubal pathology (OR 13.9). A forward stepwise logistic regression analysis and bootstrap analysis revealed that only adding the hs-CRP test significantly improved the diagnostic performance of the *C. trachomatis* IgG test (OR of test combination 39.7). Adding the *C. trachomatis* IgA test to the above-mentioned test combination led to an OR of 51.6, but the increase in OR was not statistically significant as compared to the combination *C. trachomatis* IgG/hs-CRP (OR 39.7). Combining three or four tests led to 100% specificity, but sensitivity decreased to 15%. In order to limit the number of data in Table II, we have only shown data on test combinations which included a positive *C. trachomatis* IgG test (i.e. the best single test).

Table II. The predictive value of single tests as well as combinations of tests including a positive *C. trachomatis*-IgG for distal tubal pathology.

No. of tests performed	Ctr-IgG[a]	Ctr-IgA[b]	cHSP 60-IgG[c]	hs -CRP[d]	No. of patients with positive test result	No. of patients with positive test result and DTP	Sens (%)	Spec (%)	PPV (%)	NPV (%)	OR	95% CI
One test	+				52	32	54	92	62	90	13.9[e]	7.0-27.5
		+			42	21	36	92	50	86	6.1[f]	3.1-12.3
			+		68	30	51	85	44	88	5.9[f]	3.2-10.9
				+	127	32	54	63	25	85	2.0[f]	1.1-3.5
Two tests	+	+			27	17	29	96	63	85	9.9	4.2-23.0
	+		+		41	28	47	95	68	89	16.7	7.9-35.7
	+			+	22	19	32	99	86	86	39.7[f]	11.2-140.5
Three tests	+	+	+		22	15	25	97	68	85	12.0	4.6-31.2
	+	+		+	11	10	17	100	91	84	51.6[f]	6.5-412.6
	+		+	+	16	16	27	100	100	86	∞	–
Four tests	+	+	+	+	9	9	15	100	100	84	∞	–

[a] Threshold titre for a positive test: 32; [b] Threshold index for a positive test: 1.4; [c] Threshold index for a positive test: 1.11; [d] Threshold concentration for a positive test: 1.0–10.0 mg/L; [e] versus [f] $P < 0.05$ (only *P*-values of Ctr-IgG versus the other single tests and test combinations were calculated). Abbreviations: cHSP60=chlamydia heat shock protein 60; CI= confidence interval; Ctr= *C. trachomatis*; DTP = distal tubal pathology; hs-CRP= high-sensitivity C-reactive protein; NPV= negative predictive value; OR= odds ratio; PPV= positive predictive value; sens = sensitivity; spec = specificity.

DISCUSSION

In this study, we hypothesized that the course of *C. trachomatis* infections is related to the risk of tubal damage. The natural course of *C. trachomatis* infections, duration of exposure to the micro-organism and re-infection rates are difficult to study, since *C. trachomatis* infections often remain asymptomatic, and consequently the onset of the infection is generally unknown. It is important, however, to identify subfertile women with persistent *C. trachomatis* infections, since they are supposed to have the highest risk of tubal pathology. In the present study, we have tested three serological markers of persistent infections in a cohort of 313 subfertile women. Our results indicate that persistent *C. trachomatis* infections play an important role in the development of tubal pathology.

IgA antibodies have been associated with chronic inflammation and infection. With respect to chlamydia, previous studies have demonstrated an association between *C. pneumoniae* IgA antibodies and its chronic sequelae, e.g. respiratory and cardiovascular morbidity. Falck *et al.* (2002) have found that the prevalence of symptoms of chronic respiratory tract disease increases parallel to the increase in *C. pneumoniae* IgA titre. In patients with coronary symptoms, a positive *C. pneumoniae* IgA titre significantly increases the risk of myocardial injury (OR 1.95) (Wong *et al.*, 2002). It has been suggested that serum IgA antibodies, as compared to IgG antibod-

ies, may be more reliable markers of persistent *C. pneumoniae* infections (Saikku, 1999).

cHSP60 is a chlamydia genus-specific protein, serving as a strong antigenic target for the immune system (Morrison *et al.*, 1989, Kaufmann, 1990). It has been suggested that antibodies to cHSP60 are markers of chronic inflammation (Kaufmann, 1990). Studies have shown a strong association between anti-cHSP60 antibodies and tubal factor subfertility. Anti-cHSP60 antibodies are significantly more prevalent in subfertile women with tubal disease (44–76%) as compared to those without tubal disease (8–19%) (Freidank *et al.*, 1995, Claman *et al.*, 1997). Among subfertile women with antibodies to *C. trachomatis*, anti-cHSP60 antibodies are significantly more prevalent in women with tubal pathology (76–81%) as compared to those without tubal pathology (0–43%) (Toye *et al.*, 1993, Arno *et al.*, 1995).

CRP is an acute phase protein. Slightly raised CRP concentrations, but still within the normal range, are known indicators of chronic inflammation. Research on the pathophysiology of coronary heart disease has shown that the association between *C. pneumoniae* infections and the risk of cardiovascular events is stronger if CRP is slightly raised, but within the normal range (Gattone *et al.*, 2001). As compared to patients without *C. pneumoniae* antibodies and a low CRP, the risk of coronary events increased when *C. pneumoniae* antibodies were present (OR 1.22; 95% CI 0.74–2.01), but increased even more when both *C. pneumoniae* antibodies and a slightly elevated CRP were present (OR 5.40; 95% CI 2.35–12.43) (Roivainen *et al.*, 2000). Serum CRP levels were significantly higher in patients with *C. pneumoniae*-infected atherosclerotic plaques (8 mg/L) as compared to patients with non-infected atherosclerotic plaques (undetectable CRP) (Johnston *et al.*, 2001). The role of CRP in tubal factor subfertility has not yet been studied.

In the present study, all evaluated serological markers of persistent infections were significantly more prevalent in women with tubal pathology as compared to women without tubal pathology. However, as single tests, the markers of persistent infections performed poorly as compared to the current screening test for tubal pathology (IgG to *C. trachomatis*). Odds ratios of IgA antibodies to *C. trachomatis* (6.1), IgG antibodies to cHSP60 (5.9) and hs-CRP (2.0) were significantly lower as compared to IgG antibodies to *C. trachomatis* (13.9).

The low OR of the *C. trachomatis* IgA and cHSP60 IgG tests might be explained by these antibodies being poorer markers of chronic inflammation than is currently presumed. The significantly lower OR of the cHSP60 IgG test, as compared to the *C. trachomatis* IgG test, might be explained by cross-reaction with the highly similar *C. pneumoniae*. Anti-*C. pneumoniae* antibodies are highly prevalent in subfertile women (detectable in 83.1% of women with distal tubal pathology and in 72.8% of women without distal tubal pathology), and are not associated with tubal disease (Den Hartog *et al.*, 2004). The manufacturer of the cHSP60 IgG test mentions that cross-reaction with other chlamydia species may occur, due to the homology of >

95% between cHSP60 of the different species. The low OR of the hs-CRP test as a single test might be explained by CRP being a general, and not a chlamydia-specific, marker of chronic inflammation.

A forward stepwise logistic regression analysis was performed in order to determine if the prognostic value of the best single test (i.e. *C. trachomatis* IgG) could be significantly improved by adding one or more tests. Only combining the *C. trachomatis* IgG test and the hs-CRP test resulted in a significantly higher OR (39.7) as compared to the *C. trachomatis* IgG test only (13.9). Measuring *C. trachomatis* IgG antibodies (markers of a previous infection), in combination with hs-CRP (a marker of the course of the infection), seems to identify a subset of subfertile women with the highest risk of persistent infections and distal tubal pathology. These results are comparable to previous studies, in which risk factors for cardiovascular disease were studied. In these studies, the association between *C. pneumoniae* and cardiovascular disease, which is commonly known, is stronger when elevated CRP levels, but within the normal range, are also detectable (Roivainen *et al.*, 2000, Gattone *et al.*, 2001, Johnston *et al.*, 2001). Using the forward stepwise logistic regression model, no significant increase in test performance was noted when adding a third and fourth test to the combination *C. trachomatis* IgG/hs-CRP.

A limitation of this study is selection and referral bias. Only women who had undergone a laparoscopy with tubal testing, which is the reference standard in diagnosing tubal pathology, were included in the present study. This inclusion criterion will cause selection bias, as has been discussed previously (Den Hartog *et al.*, 2004). This bias will be worsened by referral bias, since the *C. trachomatis* IgG test was used in the decision regarding who received a laparoscopy. However, it is hard to prevent selection and referral bias in a study like ours, since a laparoscopy, which has costs and risks, is not a routine procedure in all subfertility patients.

The clinical purpose of serial testing is to find a combination of tests which can estimate the risk of tubal disease more accurately. The ultimate goal would be a test combination with a PPV and NPV of 100%. In these cases, invasive tubal testing may no longer be indicated. This goal has not yet been achieved in the present study. However, if our results could be confirmed in a larger study, the test combination *C. trachomatis* IgG/hs-CRP might be a better screening method for tubal pathology as compared to the current method (*C. trachomatis* IgG only). In daily practice, all *C. trachomatis*-positive samples could be retested with the hs-CRP test, in order to identify those women who are at highest risk of a persistent *C. trachomatis* infection and tubal disease.

In summary, we hypothesized that the risk of tubal pathology is increased in subfertile women with persistent *C. trachomatis* infections. We have studied serological markers of persistent infections in subfertile women. All evaluated serological markers of persistent *C. trachomatis* infections are significantly more common in subfertile women with tubal pathology as compared to women without tubal pa-

thology. *C. trachomatis* IgG-positive subfertile women with raised CRP concentrations, but still within the normal range, are supposed to have persistent *C. trachomatis* infections and are at highest risk of tubal pathology.

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Chapter 3

The role of chlamydia genus-specific and species-specific IgG antibody testing in predicting tubal disease in subfertile women

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ABSTRACT

Background
We evaluated whether measuring chlamydia genus- and species-specific immunoglobulin (Ig) G antibodies might improve the predictive value of *Chlamydia* (*C.*) *trachomatis* antibody testing (CAT) in screening for distal tubal pathology (DTP).

Methods
Serum of 313 subfertile women was tested for the presence of species-specific antibodies to *C. trachomatis*, *C. pneumoniae* and *C. psittaci* and genus-specific antibodies to chlamydia lipopolysaccharide (LPS). Only patients who had undergone a laparoscopy with tubal testing, to assess the grade of DTP, were included in this study.

Results
The presence of *C. trachomatis* antibodies was the only independent predictor for DTP. The predictive value of CAT for DTP could not be improved by adding test results of *C. pneumoniae* or LPS antibody testing. The role of *C. psittaci* could not be evaluated, due to the absence of *C. psittaci* IgG-positive patients in our cohort.

Conclusions
In spite of the high interspecies homology, *C. pneumoniae* does not contribute to the development of DTP. Anti-LPS antibodies, which are considered to be markers for ongoing infections, do not identify *C. trachomatis*-positive subfertile women who are at highest risk of DTP. The high prevalence of anti-LPS antibodies in *C. trachomatis*-positive subfertile women may suggest that *C. trachomatis* remains more active in the upper genital tract than currently is presumed.

INTRODUCTION

Chlamydia species which can cause infections in humans are *Chlamydia* (*C.*) *pneumoniae*, *C. psittaci* and *C. trachomatis*. *C. pneumoniae* is a widespread pathogen that causes respiratory tract infections, and is associated with asthma (Hahn *et al*., 1991) and cardiovascular disease (Saikku *et al*., 1988). The prevalence of immunoglobulin (Ig) G antibodies to *C. pneumoniae* in subfertile women ranges from 56 to 76%, regardless of the cause of subfertility (Freidank *et al*., 1995, Gijsen *et al*., 2001), and is comparable to the prevalence in healthy males and females (63-74%) (Karvonen *et al*., 1994, Wong *et al*., 1999).

C. psittaci is most prevalent in birds (psittacosis), but can also infect humans and result in pneumonia. The prevalence of *C. psittaci* IgG antibodies in serum depends on population characteristics. A prevalence of 39% is reported in pigeon fanciers, a risk group for acquiring psittacosis (Bourke *et al*., 1992). In healthy males and females, as well as in patients attending a genitourinary clinic, the prevalence of IgG antibodies to *C. psittaci* is 0.1% (Moss *et al*., 1993, Wong *et al*., 1999).

The role of *C. trachomatis* in tubal factor subfertility is well established. The prevalence of *C. trachomatis* IgG antibodies is significantly higher in subfertile women with bilaterally occluded tubes (52-73%) as compared to subfertile women without tubal pathology (17-24%) (Freidank *et al*., 1995, Gijsen *et al*., 2001). Healthy (supposedly fertile) female controls have the lowest prevalence (9-10%) (Freidank *et al*., 1995).

A widely used screening method for tubal factor subfertility is chlamydia antibody testing (CAT) by measuring serum IgG antibodies to *C. trachomatis*. However, its predictive value is limited, due to false-positive and false-negative test results (Mol *et al*., 1997). The predictive value of CAT might be improved by using other serological markers for chlamydia-associated tubal damage.

First, it has been questioned whether *C. pneumoniae* and *C. psittaci*, following a primary *C. trachomatis* infection, may play a role in the development of tubal pathology. In a previous study, tubal factor subfertility seemed to be more common in subfertile women with IgG antibodies to both *C. trachomatis* and *C. pneumoniae* (49%) as compared to those with antibodies to *C. trachomatis* only (30%), but the difference was not statistically significant (Gijsen *et al*., 2001). Based on the findings of our previous study, we hypothesize that other chlamydia species, besides *C. trachomatis*, may play a role in the development of tubal factor subfertility.

Furthermore, it has been assumed that repeated exposure to *C. trachomatis* (by reactivation or reinfection) is an important risk factor for the development of tubal damage (Grayston *et al*., 1985, Patton *et al*., 1994). A previous study suggested that anti-lipopolysaccharide (LPS) antibodies are indicators of ongoing chlamydia infections (Tuuminen *et al*., 2000). Therefore, our second hypothesis is that serum IgG

antibodies to chlamydia LPS may be useful (as potential markers of ongoing *C. trachomatis* infections) in predicting the risk of distal tubal pathology.
In the present study, we used a commercially available micro-immunofluorescence (MIF) test to detect species-specific antibodies to *C. trachomatis*, *C. pneumoniae* and *C. psittaci*. A commercially available enzyme-linked immunosorbent assay (ELISA) was used for the detection of genus-specific antibodies to chlamydia LPS. We evaluated the prevalence of species-specific IgG antibodies to *C. trachomatis*, *C. pneumoniae* and *C. psittaci* respectively, as well as genus-specific IgG antibodies to chlamydia LPS in subfertile women who had undergone a laparoscopy with tubal testing. The serological data were correlated with the presence of distal tubal pathology at laparoscopy.

MATERIALS AND METHODS

The study was performed in subfertile women who entered our clinic between December 1990 and November 2000. As part of the fertility work-up, blood was drawn from all patients at their initial visit for CAT, using a MIF test (Biomérieux, the Netherlands). All spare sera were cryopreserved. Patients with a negative CAT and an otherwise normal fertility work-up underwent a hysterosalpingography (HSG) to evaluate the tubal status. If the HSG showed abnormalities, or if they did not conceive within 6 months after the HSG, a laparoscopy with tubal testing was performed. Patients with a positive CAT underwent a laparoscopy with tubal testing immediately after the fertility work-up. Only patients who had undergone a laparoscopy and tubal testing with methylene blue dye were included in the present study. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded.
For this study, the spare sera of the participating patients were thawed to perform a species-specific MIF test (AniLabsystems, Finland) and a *Chlamydia* LPS ELISA (Medac, Germany), as described below. In the present study, the MIF test by AniLabsystems was used to detect IgG antibodies to *C. trachomatis*, instead of the MIF test by Biomérieux which was used in the fertility work-up, since the test by AniLabsystems was found to predict tubal pathology more accurately than the test by Biomérieux (Land *et al*., 2003).
Two independent investigators, who were unaware of the CAT results, scored 313 successive laparoscopy reports to assess the grade of distal tubal pathology. In this study, distal tubal pathology was defined as extensive peri-adnexal adhesions and/or distal occlusion of at least one tube (Land *et al*., 1998). Subfertile women without distal tubal pathology served as controls. The controls had an unexplained subfertility, partners with mild male factor subfertility, or proximal occlusion of at least one tube.

Serological methods

IgG antibodies to *C. trachomatis*, *C. pneumoniae* and *C. psittaci* were detected using the *Chlamydia pneumoniae* IgG MIF test (AniLabsystems, Finland). For this purpose, 10 µl of the serum was diluted eight times in phosphate-buffered saline (PBS) and incubated on the microscope slides dotted with three chlamydia antigens for 30 min at 37°C in a moist chamber. The slides were washed four times with PBS and twice with distilled water and incubated with goat anti-human IgG–fluorescein isothiocyanate conjugate for 30 min at 37°C. Again the slides were washed four times with PBS and twice with distilled water. Mounting fluid was added on the slides, and a cover slip was placed on the slides. Under the microscope the slides were read. All slides were evaluated independently by two readers. In case of disagreement, which was the case in ~ 10% of all slides, the judgement of a third reader was decisive. For a quantitative determination, serial dilutions in PBS were performed. In the present study, we considered the test results of each chlamydia species as a single test. For *C. trachomatis* and *C. pneumoniae*, the cut-off titre for a positive test was 32, according to the manufacturer's instructions. According to the manufacturer's instructions, LPS was still present on the *C. psittaci* elementary bodies of the MIF test, while the LPS activity on the *C. trachomatis* and *C. pneumoniae* elementary bodies had been reduced. Therefore, the fluorescence on the *C. psittaci* spot could be due to species-specific anti-*C. psittaci* antibodies or genus-specific anti-LPS antibodies. We considered the *C. psittaci* IgG antibodies positive when the IgG titre was ≥ 2-fold than the titre of IgG antibodies to *C. trachomatis* or *C. pneumoniae*, a commonly used definition in literature (Moss *et al*., 1993, Wong *et al*., 1999).

IgG antibodies to chlamydia LPS were detected using the *Chlamydia* IgG rELISA (Medac, Germany). For this purpose, sera were diluted 1:100 in PBS and tested in microplates coated with chlamydia-specific recombinant LPS fragments. The plates were incubated for 60 min at 37°C in a humid chamber. The plates were washed three times with 200 µl PBS and tapped dry. To each well, 50 µl of conjugate (goat anti-human IgG, horseradish peroxidase-conjugated) was added and the plates were incubated for 60 min at 37°C. Again the plates were washed three times with 200 µl PBS and tapped dry. To each well, 50 µl of tetramethylbenzidine substrate was added and the plates were incubated for 30 min at 37°C. Finally, 100 µl of 0.5 mol/L sulphuric acid was added to stop the colouring reaction. The optical density of the plates was measured in a spectrophotometer at 450 nm. Threshold indexes were calculated according to the manufacturer's instructions. The threshold index for a positive test was 1.1.

Statistical methods

Characteristics of women with and without distal tubal pathology were compared using the Mann–Whitney *U*-test. For comparison of the prevalence of IgG antibodies to *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and LPS in women with and without distal tubal pathology, the χ^2-test was used. The association between chlamydia genus- and species-specific antibodies and the presence of distal tubal pathology at laparoscopy was calculated by a logistic regression analysis. The prognostic value of single testing as well as combined testing for distal tubal pathology was determined by calculating sensitivity, specificity, odds ratio (OR) and 95% confidence interval (CI). The bootstrap technique was used to test the difference between ORs (Efron and Tibshiram, 1993). $P < 0.05$ was considered statistically significant.

RESULTS

In 313 subfertile women, chlamydia IgG antibody titres in serum and laparoscopy results were available for analysis. At the start of the fertility work-up, the mean age of the women (30.6 and 31.2 years) and the mean duration of subfertility (2.4 and 2.3 years) did not differ significantly between women with and without distal tubal pathology. In total, there were 59 women (18.8%) who met the definition of distal tubal pathology, whereas 254 women (81.2%) did not have distal tubal pathology at laparoscopy. Of those 254 women without distal tubal pathology, 94.9% had patent tubes and 5.1% had proximal occlusion of at least one tube. Since proximal tubal occlusion is considered not to be associated with chlamydia disease, all 254 women without distal tubal pathology served as controls.

First, we evaluated all four tests (*C. trachomatis*, *C. pneumoniae*, *C. psittaci* and LPS) separately. Table I shows the prevalences of IgG antibodies to *C. trachomatis*, *C. pneumoniae*, *C. psittaci* and LPS in women with and without distal tubal pathology. The prevalence of species-specific IgG antibodies to *C. trachomatis* was significantly higher in women with distal tubal pathology (54.2%), as compared to women without distal tubal pathology (7.9%). Species-specific antibodies to *C. pneumoniae* were detected in 83.1% of women who had distal tubal disease, and in 72.8% of women without distal tubal disease. This difference was not statistically significant. No patients met the definition of a positive test for species-specific *C. psittaci* IgG antibodies. Genus-specific anti-LPS antibodies were detectable in 62.7% of women with tubal pathology, and in 33.9% of women without tubal pathology ($P < 0.0001$).

Using a logistic regression model, the association between *C. trachomatis* IgG antibodies and the presence of distal tubal pathology was statistically significant ($P < 0.0001$). The presence of IgG antibodies to *C. pneumoniae* ($P = 0.6$) or LPS ($P = 0.8$) was not an independent predictor for distal tubal disease.

Table I. Prevalence of IgG antibodies to *C. trachomatis* (Ctr), *C. pneumoniae* (Cpn), *C. psittaci* (Cps) and lipopolysaccharide (LPS) in women with and without distal tubal pathology (DTP).

	n	Ctr+[a]	Cpn+[a]	Cps+[b]	LPS+[c]
DTP	59	32 (54.2)[d]	49 (83.1)	0	37 (62.7)[d]
No DTP	254	20 (7.9)[e]	185 (72.8)	0	86 (33.9)[e]

Values in parentheses are percentages. [a]Threshold titre for a positive test: 32; [b]Threshold titre for a positive test: a ≥ 2-fold titre than Ctr or Cpn; [c]Threshold index for a positive test: 1.1; [d] versus [e]: $P < 0.0001$.

Secondly, the outcome of combined testing was evaluated. In combined testing, antibodies to *C. trachomatis* were measured in combination with antibodies to *C. pneumoniae* and/or LPS. The results are shown in Table II. The OR of *C. trachomatis* antibody testing was 13.9. The OR increased to 15.4 when both *C. trachomatis* and *C. pneumoniae* antibodies were detectable. When both *C. trachomatis* and LPS antibodies were present, the OR was 13.6. The highest OR (16.6) was reached in patients in whom all three antibodies were present. The increase in OR, when one or two more test results were added, as compared to testing for *C. trachomatis* antibodies only, was not statistically significant.

Table II. Predictive value of single testing as well as combined testing for distal tubal pathology.

No. of tests performed	Ctr[a]	Cpn[a]	LPS[b]	No. of patients with positive test result	Sensitivity (%)	Specificity (%)	OR	95% CI
One test	+	NA	NA	52	54.2	92.1	13.9[c]	6.6–29.2
	NA	+	NA	234	83.1	27.2	1.8[d]	0.9–4.3
	NA	NA	+	123	62.7	66.1	3.3[d]	1.8–6.2
Two tests	+	NA	+	48	50.8	92.9	13.6	6.4–29.0
	+	+	NA	46	50.8	93.7	15.4	7.1–33.8
Three tests	+	+	+	43	49.2	94.5	16.6	7.4–37.5

[a] Threshold titre for a positive test: 32; [b] Threshold index for a positive test: 1.1; [c] versus [d]: $P < 0.0001$. Abbreviations: CI = confidence interval; Cpn = *C. pneumoniae*, Ctr = *C. trachomatis*; LPS = lipopolysaccharide; NA = not analyzed; OR = odds ratio.

DISCUSSION

We evaluated whether potential alternative serological markers for distal tubal pathology might improve the predictive value of *C. trachomatis* IgG antibody testing. In the present study, serological test results were compared with the findings at laparoscopy. Therefore, only women who had undergone a laparoscopy, the reference standard in diagnosing tubal pathology, were included. This inclusion criterion will cause selection bias, which will influence the prevalence of tubal pathology, but which is hard to prevent in clinical studies. In our cohort, the prevalence of tubal

pathology will be higher as compared to an unselected population. The prevalence of tubal pathology in our tertiary care population (18.8%), however, is comparable to findings reported from other tertiary care centres (Collins *et al*., 1995).

The first aim of this study was to evaluate the role of different chlamydia species in the development of distal tubal pathology, since there is a high interspecies homology in various chlamydia antigens, such as chlamydia heat shock protein 60 (cHSP60) (Kikuta *et al*., 1991) and LPS (Caldwell and Hitchcock, 1984). The potential role of *C. pneumoniae* or *C. psittaci* infections in the development of tubal disease, following a primary *C. trachomatis* infection, could be explained by a chlamydia genus-specific auto-immune inflammatory response, leading to tissue damage. Such a mechanism has previously been suggested by Wick *et al*. (2001), who studied the role of *C. pneumoniae* in the development of atherosclerosis. The immune response to *C. pneumoniae*, a highly prevalent micro-organism, does not normally result in vascular damage. It is hypothesized that in the presence of a stressor (e.g. hypertension), the vascular endothelial cells express human heat shock protein 60 (hHSP60) on their surface, which may become a target for antibodies initially directed against the highly similar cHSP60. This may lead to destruction of the endothelial cells and the development of atherosclerotic lesions (Wick *et al*., 2001). Extrapolating this hypothesis to the development of tubal damage, we hypothesize that the basic condition for the development of tubal pathology is a primary (silent) *C. trachomatis* infection (stressor), leading to the expression of hHSP60 on the tubal epithelium. During an infection, genus-specific antibodies to cHSP60 are produced, which can cross-react with hHSP60 on the epithelial cells in the tubes, leading to epithelial damage and subsequently to tubal pathology. Since cHSP60 is genus-specific, the auto-immune response may be induced by all chlamydia species.

To evaluate the role of different chlamydia species in the development of distal tubal pathology, we first studied the prevalence of antibodies to the three chlamydia species in our cohort. In subfertile women with and without distal tubal pathology, the prevalences of antibodies to *C. trachomatis*, *C. pneumoniae* and *C. psittaci* (Table I) were comparable to the prevalences as reported earlier (Moss *et al*., 1993, Freidank *et al*., 1995, Wong *et al*., 1999, Gijsen *et al*., 2001).

The association between serum IgG antibodies to *C. trachomatis* and tubal pathology is commonly known (Punnonen *et al*., 1979, meta-analysis by Mol *et al*., 1997), and was confirmed in the present study. A significant additive role of *C. pneumoniae* in the development of distal tubal pathology could not be found, and, in spite of enlargment of the cohort, the findings of our previous study (Gijsen *et al*., 2001) could not be confirmed. The role of *C. psittaci* infections could not be evaluated in the present study.

The second aim of this study was to evaluate genus-specific IgG antibodies to chlamydia LPS, as potential serological markers of repeated exposure to *C. trachomatis*. LPS is an outer membrane component shared by all three chlamydia species (Cald-

well and Hitchcock, 1984), which has antigenic capacities comparable to the major outer membrane protein (MOMP). It has been reported previously that genus-specific anti-LPS antibodies increase rapidly in the early phases of infections, whereas more specific anti-MOMP antibodies are produced at a later stage (Ekman *et al*., 1993). It is assumed that repeated exposure to *C. trachomatis* is an important risk factor for the development of tubal damage (Grayston *et al*., 1985, Patton *et al*., 1994). Repeated exposure to pathogens causes repeated stimulation of the immune system, and subsequently may cause a continuously high level of anti-LPS antibodies. A study of Tuuminen *et al*. (2000) supports this hypothesis. Repeated stimulation might be caused by an endogenous reactivation of persistent *C. trachomatis* micro-organisms or by an exogenous reinfection with *C. trachomatis*.

As shown in Table I, the prevalence of anti-LPS antibodies was significantly higher in women with distal tubal pathology (62.7%) as compared to women without distal tubal pathology (33.9%). Further analysis revealed a remarkable overlap between women with IgG antibodies to *C. trachomatis* and to LPS: in 92.3% of the women with *C. trachomatis* antibodies, anti-LPS antibodies were detectable (data not shown). However, the presence of anti-LPS antibodies was no independent predictor for tubal disease. The low OR of the LPS-only test (3.3) might be explained by the high prevalence of *C. pneumoniae* antibodies in our cohort, causing a positive LPS test in women who do not necessarily have *C. trachomatis* IgG antibodies and tubal pathology. We did not find a significant additive role of anti-LPS antibodies in predicting the risk of tubal disease.

The high prevalence of anti-LPS antibodies in subfertile women with distal tubal pathology (62.7%) is in agreement with findings in previous studies, in which other markers of ongoing infections were demonstrated in the upper genital tract of subfertile women with late sequelae of *C. trachomatis* (Gérard *et al*., 1998, Kinnunen *et al*., 2002). The high prevalence of anti-LPS antibodies in *C. trachomatis*-positive subfertile women without distal tubal pathology (33.9%), however, suggests that *C. trachomatis* may remain more active in the genital tract than is currently presumed. These women may also have viable micro-organisms in the upper genital tract, which may cause minimal tubal epithelial damage or silent endometritis, and may compromise their fertility, despite normal findings at laparoscopy.

In summary, we evaluated whether additional determination of IgG antibodies to *C. pneumoniae*, *C. psittaci* and chlamydia LPS might improve the predictive value of *C. trachomatis* IgG antibody testing in screening for tubal factor subfertility. Nonetheless, in spite of the high interspecies homology, *C. pneumoniae* does not seem to contribute to the development of distal tubal pathology. The role of *C. psittaci* cannot be evaluated, due to the absence of *C. psittaci* IgG-positive patients in our cohort. Although anti-LPS antibodies are considered as markers for chronic inflammation, their presence is not useful in selecting a subset of *C. trachomatis*-positive subfertile women which is most likely to have tubal damage. The high prevalence of

anti-LPS antibodies in our cohort suggests that *C. trachomatis* may remain more active in the upper genital tract than is currently presumed. *C. trachomatis*-positive subfertile women with anti-LPS antibodies, but without tubal disease, may have a mild chronic infection, which may also compromise their fertility.

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Chapter 4

Screening strategies for tubal factor subfertility

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ABSTRACT

Background
Different screening strategies exist to estimate the risk of tubal factor subfertility preceding laparoscopy. Three screening strategies, comprising *Chlamydia trachomatis* IgG antibody testing (CAT), high-sensitivity C-reactive protein (hs-CRP) and hysterosalpingography (HSG), were explored using laparoscopy as reference standard and the occurrence of a spontaneous pregnancy as surrogate marker for the absence of tubal pathology.

Methods
In this observational study, 642 subfertile women, who underwent tubal testing, participated. Data on serological testing, HSG, laparoscopy and interval conception were collected. Multiple imputations were used to compensate for missing data.

Results
Strategy A (HSG) has limited value in estimating the risk of tubal pathology. Strategy B (CAT $\rightarrow$ HSG) shows that CAT significantly discerns patients with a high versus low risk of tubal pathology, whereas HSG following CAT has no additional value. Strategy C (CAT $\rightarrow$ hs-CRP $\rightarrow$ HSG) demonstrates that hs-CRP may be valuable in CAT-positive patients only and HSG has no additional value.

Conclusions
CAT is proposed as first screening test for tubal factor subfertility. In CAT-negative women, HSG may be performed because of its high specificity and fertility-enhancing effect. In CAT-positive women, hs-CRP seems promising, whereas HSG has no additional value. The position and timing of laparoscopy deserves critical reappraisal.

INTRODUCTION

The reference standard for diagnosing tubal factor subfertility is laparoscopy with tubal dye testing, by which tubal patency and the presence of peri-adnexal adhesions and endometriosis can be assessed. It also provides additional information on uterine malformations. Laparoscopy has several disadvantages, e.g. it is an invasive and expensive procedure requiring general anaesthesia, and it holds a 1.5% risk of surgical complications (Chapron *et al.*, 1998). Furthermore, operating facilities may not be easily available in every clinic. Owing to these disadvantages, laparoscopy is unsuitable for routine application in subfertile women on a large scale. Obtaining a reliable estimate of the risk of tubal pathology preceding laparoscopy would allow for selecting only high-risk patients for this procedure. The chance of finding tubal pathology at laparoscopy can be estimated preoperatively by applying screening tests. Among different countries the preferred screening method to determine the risk of tubal pathology varies (Portuondo *et al.*, 1984, Helmerhorst *et al.*, 1995, Balasch, 2000, Mol *et al.*, 2001). Commonly used and well-evaluated modalities to screen for tubal pathology in the fertility evaluation are serum *Chlamydia* (*C.*) *trachomatis* IgG antibody testing (CAT) and hysterosalpingography (HSG) (Swart *et al.*, 1995, Mol *et al.*, 1997, Mol *et al.*, 2001).

CAT is a serological marker of a previous *C. trachomatis* infection, but does not reflect the course of the infection. Because mainly persistent *C. trachomatis* infections, rather than cleared infections, are associated with an increased risk of tubal pathology (Grayston *et al.*, 1985, Patton *et al.*, 1994), the combination of CAT and high-sensitivity C-reactive protein (hs-CRP), reflecting a previous *C. trachomatis* infection and persistence of the micro-organism respectively, has been proposed as a valuable set of markers for identifying subfertile women at highest risk of persistent *C. trachomatis* infection and the ensuing tubal pathology (Den Hartog *et al.*, 2005). In the present study, HSG is considered as a screening test (as opposed to a diagnostic test) because of its lower performance in diagnosing tubal pathology and predicting future fertility as compared to laparoscopy (Swart *et al.*, 1995, Mol *et al.*, 1999) and because at present, in many clinics, HSG is used to differentiate between patients who qualify for further tubal testing and those who do not. The role of HSG in the investigation of the subfertile couple covers more than testing tubal patency only, because it also provides additional information on uterine malformations and intracavitary pathology.

No consensus exists on which screening test (or series of screening tests) is to be preferred for assessing the risk of tubal pathology in subfertile women. In the present observational study, we evaluated different screening strategies in predicting tubal pathology, in order to develop a useful screening strategy in the diagnostic work-up of subfertile couples. For this purpose, three different screening strategies comprising the test modalities CAT, hs-CRP and HSG were explored in a population

of subfertile women by constructing decision tables (Glasziou and Hilden, 1986), and using laparoscopy as reference standard and the occurrence of a spontaneous pregnancy as a surrogate marker for the absence of tubal pathology.

MATERIALS AND METHODS

Study population

The study population consisted of 642 women who visited the Maastricht University Medical Centre Fertility Clinic because of subfertility between December 1990 and May 2004, and who consecutively underwent tubal testing (HSG and/or laparoscopy) as part of their fertility evaluation. In all women, blood was drawn at their initial visit to measure *C. trachomatis* IgG antibodies using CAT. Spare serum samples were cryopreserved and stored. In patients with a negative CAT and an otherwise normal basic fertility evaluation, tubal status was evaluated initially by HSG. If HSG showed abnormalities, or if patients did not conceive in the 6 months following HSG, the fertility evaluation was concluded by laparoscopy with tubal testing using methylene blue dye. In patients with a positive CAT, no HSG was performed, but tubal status was evaluated primarily by laparoscopy. Patients with a history of pelvic surgery (except for an uneventful appendectomy or Caesarean section) or pelvic inflammatory disease, and patients with suspected severe endometriosis, based on history and findings at pelvic examination, directly underwent laparoscopy and were excluded from the present study.
In the Netherlands, for retrospective analysis of anonymized patient data and stored sera, no ethical committee approval is required. In the Fertility Clinic of the Maastricht University Medical Centre, all couples are informed at intake about possible use of their anonymized data and stored sera for research purposes, and a “no objection procedure” is followed. Only patients having not objected participated in the present study.

CAT

For the present study, stored serum samples of 475 patients were available and retested for CAT by using an enzyme-linked immunosorbent assay (ELISA) (*C. trachomatis* IgG pELISA, Medac, Germany). The test was performed according to the manufacturer’s instructions. The cut-off level used for a positive test was > 1.1.

hs-CRP

In each of the 475 available stored samples, hs-CRP was determined using CRP ELISA (DiaMed Eurogen, Belgium). A high-sensitivity test was used in order to reliably detect low CRP concentrations. The test was used according to the manufacturers' instructions. Hs-CRP levels between 1.0 and 10.0 mg/L were considered positive, whereas hs-CRP levels < 1.0 and > 10.0 mg/L were considered negative (Den Hartog *et al.*, 2005).

HSG

In 424 patients, HSG was performed using oil-soluble contrast medium. The procedure was recorded on videotape, and radiographs were made during the procedure and 24 h afterwards to visualize the residual intra-abdominal spread of the contrast medium. The video recordings and radiographs of all HSGs were discussed in a consensus meeting. Besides tubal patency and intra-abdominal spread of contrast medium, uterine malformations and intracavitary pathology were assessed. HSG was considered normal when both tubes were patent and contrast medium had spread normally after 24 h, or when only a uterine malformation or minor intracavitary pathology (both unlikely to be related to the patient's fertility) was found. HSG was considered abnormal when both tubes were occluded and no spill of contrast medium was seen in the abdominal cavity after 24 h. HSG was considered inconclusive in all other cases (i.e. unilateral tubal occlusion, uncertain unilateral or bilateral tubal patency, high pressure needed to fill the tubes, pockets of contrast medium other than in the pouch of Douglas, or when the procedure had been abandoned).

Laparoscopy with tubal testing

In 355 patients, laparoscopy with tubal testing using methylene blue dye was performed. Tubal patency and the presence of peri-adnexal adhesions and endometriosis were assessed. For endometriosis, the 1996 revised American Society for Reproductive Medicine (rASRM) classification was used (The American Society for Reproductive Medicine, 1997). Laparoscopies were performed by gynaecologists who were not blinded for CAT and/or HSG results. For the present study, all laparoscopy reports were scored independently by two investigators (J.A.L. and J.L.H.E.) who were unaware of CAT and/or HSG results. In cases of disagreement, consensus was reached by consultation. On the basis of tubal patency and the presence of peri-adnexal adhesions and endometriosis, findings at laparoscopy were categorized into three levels according to the patient's estimated spontaneous pregnancy chance: normal spontaneous pregnancy chance (subcategories: no abnormalities and no fertility-impairing factors), reduced spontaneous pregnancy chance and

(nearly) absent spontaneous pregnancy chance respectively. Subfertile women with reduced and (nearly) absent spontaneous pregnancy chances were considered together as a group having tubal pathology, whereas those with normal spontaneous pregnancy chances were considered not to have tubal pathology. This categorization was based on the Hull and Rutherford classification (Rutherford and Jenkins, 2002) and on our experts' group opinion (J.A.L. and J.L.H.E.). Table I reflects the classification of the findings at laparoscopy.

Table I. Overview of the classification of tubal pathology and estimated spontaneous pregnancy chance, respectively, based on findings at laparoscopy (if available) or on interval conception.

	n	Estimated spontaneous pregnancy chance	Findings at laparoscopy, based on tubal patency and the presence of peri-adnexal adhesions and endometriosis
No tubal pathology (n = 455)	89	Normal (no abnormalities)	Patent tubes, no adhesions, no endometriosis
	161	Normal (no fertility-impairing factors)	Patent tubes, no/any adhesions, no/Stage I endometriosis Unilateral tubal occlusion, no adhesions, no/Stage I endometriosis
	205	Normal	No laparoscopy because of interval conception before laparoscopy (spontaneous or treatment-dependent conception, IVF-pregnancies not included)
Tubal pathology (n = 105)	58	Reduced	Patent tubes, any/extensive unilateral adhesions, no/Stage I/II/III endometriosis Patent tubes (high pressure needed), no/any/extensive adhesions, no/Stage I endometriosis Unilateral tubal occlusion with normal contralateral side, no/any/extensive unilateral adhesions, no/Stage I endometriosis
	47	(Nearly) absent	Patent tubes, extensive bilateral adhesions, no/Stage I/II/III endometriosis Patent tubes (very high pressure needed), no/any/extensive adhesions, no/Stage I/II/III endometriosis Unilateral tubal occlusion with suboptimal contralateral side, no/any/extensive adhesions, no/Stage I/II/III endometriosis Bilateral tubal occlusion, no/any/extensive adhesions, no/Stage I/II/III endometriosis
Unknown (n = 82)	82	Unknown	No laparoscopy, no interval conception (active or passive discontinuation of fertility evaluation or referral for IVF)
TOTAL	642		

No laparoscopy because of interval conception

No laparoscopy was performed in 205 patients, because they conceived either spontaneously or following treatment (other than IVF) before laparoscopy. Interval conception was used as a surrogate marker for the absence of tubal pathology, and therefore these 205 patients were categorized as having no tubal pathology (Table

I), although it cannot be ruled out that minor degrees of tubal pathology may have been present. Patients who conceived by IVF were not included in this subgroup, because IVF-pregnancies are independent of tubal function.

No laparoscopy and no interval conception

In 82 patients, no laparoscopy was performed and no pregnancy occurred either spontaneously or after treatment (other than IVF) following HSG, because these patients decided to stop treatment, were advised to stop treatment or were referred for IVF. In this subgroup of patients, no reference standard was available and neither was a surrogate outcome.

Statistical methods

In the study population of 642 women, serum samples were missing from 167 patients, HSG was not performed in 218 patients and no laparoscopy or interval conception occurred in 82 women (Figure I). Exclusion of all patients with an incomplete data set would result in an inefficient reduction of the population size and might introduce bias if the excluded group were a non-randomly selected subsample from the entire population. Therefore, we used an alternative approach based on multiple imputations (Van Buuren *et al.*, 1999, Altman and Bland, 2007, Horton and Kleinman, 2007). Five imputations were performed (A.G.H.K.), in which the missing data on CAT, hs-CRP and HSG were imputed as described extensively by Van Buuren *et al.* (1999). Imputed data were calculated by using all available data of the entire population. Each of the five completed data sets was analyzed, and the results were integrated into a final result (C.M.J.G.L.) (Van Buuren *et al.*, 1999, Horton and Kleinman, 2007).
In 82 patients, no reference standard was available (Figure I). Excluding this subgroup might also introduce selection bias. With a logistic regression model, we determined which subgroups were underrepresented and in all analyses, weights were used to compensate for this underrepresentation (Horton and Kleinman, 2007).

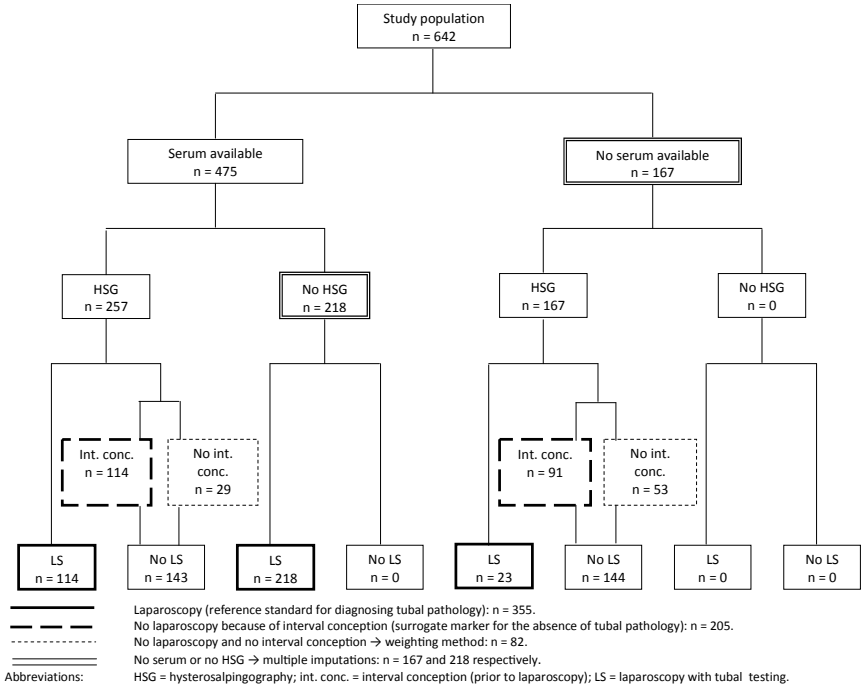

Figure I. Overview of the obtained data.

Screening strategies

Three screening strategies for tubal factor subfertility were evaluated comprising the test modalities CAT, hs-CRP and HSG. In strategy A, HSG was performed. In strategy B, CAT and HSG were performed consecutively. In strategy C, CAT, hs-CRP and HSG were performed consecutively. All strategies were designed in a manner that tests were performed in order of increasing invasiveness. Laparoscopy was used as reference standard for the presence or absence of tubal pathology, and the occurrence of a spontaneous pregnancy was used as surrogate marker for the absence of tubal pathology. For all strategies, decision tables were constructed, in which all possible combinations of test results were listed (Glasziou and Hilden, 1986). Subsequently, the posterior probabilities of tubal pathology and 95% confidence intervals were calculated for all combinations using a logistic regression model. Furthermore, with this model, the significance of diagnostic coefficients was tested.

RESULTS

Population characteristics

The study population consisted of 642 subfertile women who attended our Fertility Clinic and who consecutively underwent tubal testing by HSG and/or laparoscopy. In 71%, subfertility was primary and in 28%, it was secondary. In 1%, this information was not available. At intake, the median age was 31 years (range 19-41) and the median duration of subfertility was 17 months (range 0-162). Serum samples were obtained at the initial visit, and spare samples of 475 women were available for CAT and hs-CRP testing. Of these 475 women, CAT was negative in 88% and positive in 12% of patients. Hs-CRP was negative in 76% and positive in 24% of patients. HSG was performed in 424 women, of whom 66% had a normal HSG, 2% had an abnormal HSG and 32% had an inconclusive HSG. Laparoscopy was performed in 355 women, of whom 70% had no tubal pathology and 30% had tubal pathology. In 205 women, no laparoscopy was performed because of interval conception, and these patients were considered not to have tubal pathology. The median duration between initial visit and HSG was 4 months (range 0-59). The median duration between HSG and laparoscopy was 8 months (range 1-46). The median duration between initial visit and laparoscopy was 10 months (range 0-71). Figure I shows an overview of the obtained data (number of serum samples, HSG, laparoscopy and spontaneous pregnancies), the imputed data and the subgroup for which a weighted analysis was performed.

Screening strategies

Figure II shows the results of screening strategy A (HSG). Figure III shows the results of screening strategy B (CAT $\rightarrow$ HSG). Figure IV shows the results of screening strategy C (CAT $\rightarrow$ hs-CRP $\rightarrow$ HSG). The diagnostic performance of HSG, as expressed by the coefficients in the logistic regression model, was not statistically significant in all three strategies (*P*-values between 0.4 and 0.8). Moreover, the diagnostic performance of CAT in strategy B and C ($P < 0.0001$) and hs-CRP in strategy C ($P < 0.003$) were highly significant.

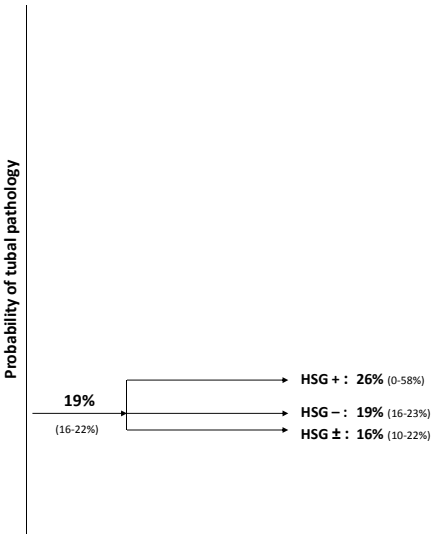

Figure II. Strategy A: HSG.
Abbreviation: HSG = hysterosalpingography. HSG—= normal, HSG ± = inconclusive; HSG + = abnormal. Values in parentheses are 95% confidence intervals.

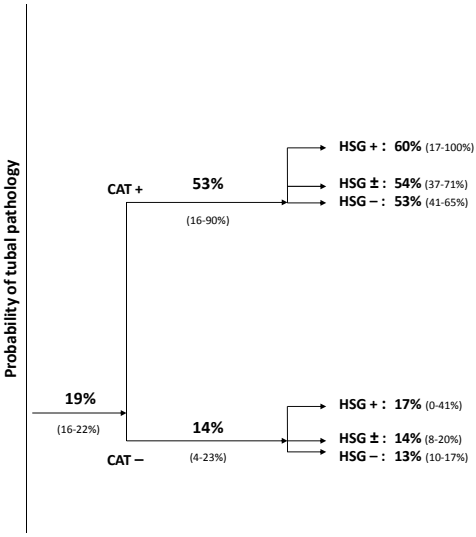

Figure III. Strategy B: CAT → HSG.
Abbreviations: CAT = *C. trachomatis* IgG antibody testing; HSG = hysterosalpingography. CAT—= negative; CAT + = positive; HSG—= normal, HSG ± = inconclusive; HSG + = abnormal. Values in parentheses are 95% confidence intervals.

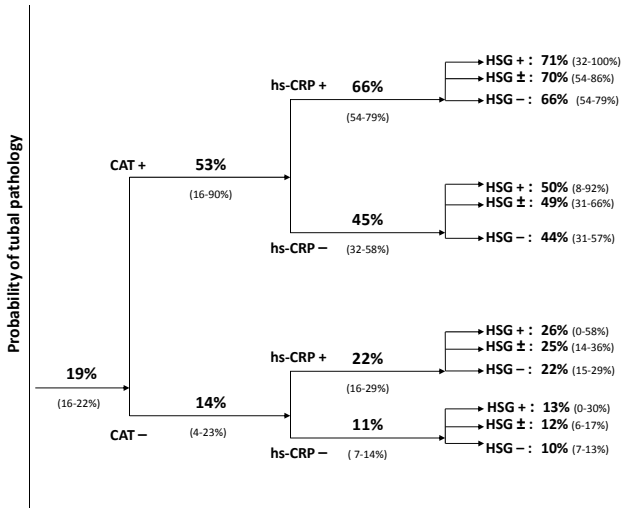

Figure IV. Strategy C: CAT → hs-CRP → HSG.
Abbreviations: CAT = *C. trachomatis* IgG antibody testing; hs-CRP = high-sensitivity C-reactive protein; HSG = hysterosalpingography. CAT—= negative; CAT + = positive; hs-CRP—= negative; hs-CRP + = positive; HSG—= normal, HSG ± = inconclusive; HSG + = abnormal. Values in parentheses are 95% confidence intervals.

Additional findings at HSG and laparoscopy

Uterine malformations and intracavitary pathology at HSG.
Uterine malformations were found in 2.4% of the HSGs (10/424): arcuate uterus (3), bicornuate uterus (3), unicornuate uterus (2), septate uterus (1) and T-shaped uterus (1). Intracavitary pathology was found or suspected in 2.1% of the HSGs (9/424): polyps (6), fibroids (2) and suspected intracavitary adhesions (1). In two patients with suspected polyps, and in the patient with suspected intracavitary adhesions, hysteroscopy was performed, in which no abnormalities were found. In the other six cases with intracavitary pathology, no hysteroscopy was performed and an expectant management was observed.

Endometriosis at laparoscopy.
Lesions suspect for endometriosis were found in 50% of all patients undergoing laparoscopy (176/355). Of these 176 patients, 88% had minimal peritoneal endometriosis without adhesions (compatible with rASRM stage I), 5% had peritoneal endometriosis with adhesions (stages II and III), 2% had ovarian endometriosis without adhesions (stages I and II) and 6% had ovarian endometriosis with adhesions (stages II and III). No stage IV endometriosis was found at laparoscopy in our study population, which was according to our expectation, because suspected severe endometriosis (based on history and findings at pelvic examination) was an exclusion criterion.

DISCUSSION

In the present study, we assessed the clinical value of three different screening strategies for assessing the risk of tubal pathology in a population of subfertile women, using serological testing (CAT and hs-CRP) and HSG as screening methods, laparoscopy as a reference standard and the occurrence of a spontaneous pregnancy as a surrogate marker for the absence of tubal pathology. The aim was to develop a useful minimally invasive screening strategy which can be used in the diagnostic work-up of subfertile couples to estimate the risk of tubal pathology preceding laparoscopy. Estimating the cost-effectiveness of the different screening strategies or analyzing cost-minimization of the screening strategies was beyond the scope of this study. Tubal pathology was defined as reduced or (nearly) absent spontaneous pregnancy chances based on tubal patency and the presence of periadnexal adhesions and endometriosis at laparoscopy. Patients with normal spontaneous pregnancy chances based on the laparoscopy reports and patients who conceived prior to laparoscopy were considered not to have tubal pathology (Table I). We have also re-analyzed our data using a more strict definition of tubal pathology, in which the subgroup with reduced spontaneous pregnancy chances was categorized as having no tubal pathology. Obviously, the pre-test probability of tubal pathology was lower (8%), but the trends in post-test probabilities as noted in the present analysis remained unchanged (data not shown). In our opinion, the definition of tubal pathology which has been used in the present study [i.e. reduced or (nearly) absent spontaneous pregnancy chances] is applicable to the average subfertile population. The more strict definition may be used in a clinical setting in which only a very high suspicion of tubal pathology, based on the results of a screening strategy, may alter clinical decision-making (e.g. in the case of limited access to operating facilities).

Strategy A: HSG

According to the fertility-guideline of the National Institute for Clinical Excellence (NICE, 2004), HSG should be offered to women who are not known to have co-morbidities (such as a history of pelvic inflammatory disease, previous ectopic pregnancy or endometriosis) to screen for tubal pathology. In women who are assumed to have co-morbidities, laparoscopy should be offered instead of HSG. Strategy A corresponds with the screening method proposed by NICE, in which only women at low risk for tubal pathology (based on the medical history) undergo HSG.
In our study population, consisting of women without known co-morbidities, the pre-test probability of tubal pathology was 19%, and the post-HSG probabilities ranged from 16 to 26% (95% CI 0—58), depending on the HSG result. It should be noted that the risk of tubal pathology did not differ between women with a normal

HSG versus an inconclusive HSG (e.g. unilateral tubal occlusion, uncertain unilateral or bilateral tubal patency, high pressure needed to fill the tubes and pockets of contrast medium other than in the pouch of Douglas), indicating that inconclusive HSGs can be categorized as normal. These results are in accordance with a study of Mol *et al.* (1999), who conclude that fertility prospects in women with unilateral tubal occlusion at HSG are only slightly lower when compared with women with bilateral tubal patency at HSG. The risk of tubal pathology in the case of a normal or inconclusive HSG is slightly lower when compared with findings of previous studies, which state that ~ 20—25% of women with normal HSG findings have tubal pathology caused by adhesions or endometriosis (Henig *et al.*, 1991, Tanahatoe *et al.*, 2003), which often remain undetected at HSG (Swart *et al.*, 1995). From our data, it can be concluded that in low pre-test risk women, a normal or inconclusive HSG is of little additional value in discerning women with a high versus low post-HSG risk of tubal pathology. No conclusion can be drawn in case of an abnormal HSG, owing to the wide 95% confidence interval of post-HSG risk of tubal pathology (0—58), although the prevalence of abnormal HSGs in our low-risk population is only 2% (9/424).

Strategy B: CAT → HSG

CAT has been introduced in the fertility evaluation as a screening test to estimate the risk of tubal pathology, because an association exists between the presence of *C. trachomatis* IgG antibodies in serum and tubal pathology (Punnonen *et al.*, 1979). The value of CAT in assessing the risk of tubal pathology is at least comparable with HSG (Dabekausen *et al.*, 1994, Perquin *et al.*, 2007). The advantage of CAT is that it is a simple, inexpensive and minimally inconvenient test method, which makes CAT recommended in subfertile women as the first screening test for tubal pathology after medical history taking (Dabekausen *et al.*, 1994, Mol *et al.*, 1997, Mol *et al.*, 2001, Veenemans and Van der Linden, 2002, Coppus *et al.*, 2007). Strategy B reflects the use of CAT prior to HSG (in the order of increasing invasiveness). Our data show that CAT accurately discerns patients with a high versus low risk of tubal pathology [post-test probability 14% (95% CI 4—23) in CAT-negative patients and 53% (95% CI 16—90) in CAT-positive patients]. Our present findings on the predictive value of CAT for tubal pathology are comparable with those reported by others (as summarized by Den Hartog *et al.*, 2006). Performing HSG after CAT does not change the probability of tubal pathology significantly. In CAT-negative women (post-CAT probability of tubal pathology 14%) the post-HSG probabilities ranged between 13 and 17% (95% CI 0—41), whereas in CAT-positive women (post-CAT probability of tubal pathology 53%) the post-HSG probabilities ranged between 53 and 60% (95% CI 17—100). From these results, it can be concluded that CAT, as a first screening test to estimate the risk of tubal pathology in the fertility work-up, is more accurate

in comparison with HSG (strategy A). In both CAT-negative and CAT-positive women, HSG following CAT has no significant additional value in estimating the risk of tubal pathology. Furthermore, it should be taken into account that HSG in CAT-positive women holds a 10% risk of post-HSG complications (e.g. fever and infection) in women with tubal pathology (Forsey *et al.*, 1990). Therefore, the value of HSG following CAT in screening for tubal factor subfertility should be critically reappraised: the post-HSG risk of tubal pathology in CAT-negative and CAT-positive women remains invariably low and high respectively, HSG is relatively contra-indicated in CAT-positive women because of increased post-infectious morbidity and CAT-positive women may benefit from early laparoscopy in order to provide a definitive diagnosis without delay.

Strategy C: CAT → hs-CRP → HSG

Because persistence of *C. trachomatis* infections is an important risk factor for tubal pathology, the additional value of serological markers of persistence has been evaluated previously (Den Hartog *et al.*, 2005). Combining CAT (as a marker of a previous *C. trachomatis* infection) and hs-CRP (as a marker of persistence) has been found a valuable test set to identify women at highest risk of tubal pathology (Den Hartog *et al.*, 2005). Strategy C reflects the use of hs-CRP following CAT and preceding HSG. Because hs-CRP is a general marker of inflammation, it is considered of importance in CAT-positive women only, in whom it may discern women with a high risk of persistence and tubal pathology (both CAT and hs-CRP positive) from those with a lower risk of persistence and tubal pathology (CAT positive and hs-CRP negative). Our results confirmed that the value of hs-CRP in assessing the risk of tubal pathology in CAT-negative women is limited. In CAT-positive women [post-CAT probability of tubal pathology 53% (95% CI 16—90)], the post-hs-CRP probabilities were 45% (95% CI 32—58) in hs-CRP-negative women and 66% (95% CI 54—79) in hs-CRP-positive women, respectively. After adding HSG as a third test, the probability of tubal pathology does not change significantly [44-71% (95% CI 8—100)]. It can be concluded that hs-CRP, which is simple, inexpensive and minimally inconvenient to patients and has previously been shown to improve the predictive value of CAT significantly (Den Hartog *et al.*, 2005), seems of additional value in estimating the risk of tubal pathology in CAT-positive subfertile women only. As discussed in strategy B, HSG has little additional value in assessing the risk of tubal pathology in CAT-negative women, and is relatively contra-indicated in CAT-positive women.

Limitations of the study

Our study has several limitations. First, serum samples from 167 patients (mainly those who visited our clinic in the early 1990s) were missing. Although CAT had

been performed as a part of their fertility evaluation, no spare samples were available for the present study. The original CAT results were obtained by a different CAT test, which has later been shown to be inferior to the CAT test used in the present study (Land *et al.*, 2003), and therefore it was decided not to use the results of the original CAT. Missing data on CAT and hs-CRP were replaced by plausible values by using multiple imputations (Van Buuren *et al.*, 1999, Altman and Bland, 2007, Horton and Kleinman, 2007). An important limitation of our study is verification bias, because the CAT result in the fertility evaluation determined whether HSG or laparoscopy was performed. In our clinic, HSG is not performed in CAT-positive women owing to the 10% risk of febrile and infectious morbidity reported in women with tubal pathology (Forsey *et al.*, 1990). Therefore, abnormal HSGs were rare (9/424 = 2%), causing wide ranges in the 95% confidence intervals. We have used multiple imputations to fill in the missing data of HSG, in order to minimize the influence of verification bias (Van Buuren *et al.*, 1999, Altman and Bland, 2007, Horton and Kleinman, 2007). This verification bias is difficult to avoid, unless one is willing to perform HSG in all subfertile women, thereby accepting its potential harmful effects, and unless one is prepared to perform both HSG and laparoscopy in all women, preferably even on the same day. Another contributor to verification bias is that only patients who underwent tubal testing (HSG and/or laparoscopy) were included. Previously, we have calculated that 70—80% of all subfertile women who start a fertility work-up will not undergo tubal testing, because of spontaneous pregnancies, immediate referral for IVF or drop out (Fiddelers *et al.*, 2005). The risk of tubal pathology will therefore be over-estimated in our study. Finally, we are aware that nowadays other methods for evaluating tubal function [such as hysterosalpingo (contrast) sonography and transvaginal hydrolaparoscopy] are used, but we have limited our study to HSG and laparoscopy because these well-evaluated tests are performed on a routine-basis in most clinics.

Omitting HSG?

It may be questioned whether HSG should still be performed in the fertility investigation, as our results indicate that HSG has limited value in the risk assessment of tubal pathology and is relatively contra-indicated in CAT-positive women. Although HSG may provide additional information on uterine malformations and intracavitary pathology, their prevalence is low (in our study 2.4% and 2.1%, respectively, which is comparable to prevalences found in the literature) (Varasteh *et al.*, 1999, Grimbizis *et al.*, 2001). The majority of these abnormalities can also be diagnosed by ultrasonography (which in most clinics is performed routinely in all subfertile patients), their presumed effect on fertility is debatable, and the effectiveness of treatment of uterine abnormalities on improving pregnancy rates has not been established (NICE, 2004). However, HSG also has advantages; therefore, omitting

HSG in CAT-negative women deserves careful consideration. The first advantage of HSG is that it has a high specificity (Swart *et al.*, 1995), i.e. HSG is accurate in confirming the absence of tubal pathology. Furthermore, tubal flushing using oil-soluble contrast medium has a positive effect on pregnancy rates (Luttjeboer *et al.*, 2007). As a consequence, omitting HSG using oil-soluble contrast medium may result in lower pregnancy rates. So far, this positive effect on pregnancy rates has not been found when water-soluble contrast medium was used (Perquin *et al.*, 2006). On the basis of these considerations, the role of HSG in the fertility evaluation of low-risk patients (i.e. CAT-negative women) deserves critical reappraisal. In CAT-positive high-risk patients, HSG should be omitted because of the 10% risk of post-HSG complications in the absence of additional value in risk assessment of tubal pathology.

Omitting laparoscopy?

Although laparoscopy is the reference standard in diagnosing tubal pathology, it may be questioned whether it should be performed routinely in all patients. Laparoscopy is still considered a useful test in women with known co-morbidities which may have compromised tubal function, such as a history of pelvic inflammatory disease (NICE, 2004). In patients with an uneventful medical history, it has been suggested that laparoscopy can be omitted after a normal HSG (Fatum *et al.*, 2002, Lavy *et al.*, 2004), although prevalence figures for tubal pathology of ~ 20—25% have been reported in women with normal HSGs (Henig *et al.*, 1991, Tanahatoe *et al.*, 2003). In our study, the prevalence of tubal pathology in CAT-negative women ranged from 10 to 22% in patients with a normal HSG and from 12 to 25% in patients with an inconclusive HSG. Omitting laparoscopy in patients with a very low (or very high) risk of tubal pathology, based on the results of screening tests, implicates that potential other causes of subfertility, such as endometriosis, remain undiagnosed and hence untreated. In our study, half of all patients undergoing laparoscopy had endometriosis, but this was limited to Stages I and II disease in over 90% of them. It is subject of discussion whether Stages I and II endometriosis reduce fertility and should therefore be treated. A meta-analysis of two randomized controlled trials showed that subfertile women with Stages I and II endometriosis benefit at least temporarily from laparoscopic surgery (Jacobson *et al.*, 2002, Kennedy *et al.*, 2005), although the number needed to treat is 24 (i.e. 24 subfertile women need to undergo laparoscopy in order to identify and treat 12 women with Stages I and II endometriosis and to achieve one additional pregnancy) (ESHRE Capri Workshop Group, 2004). The decision whether to perform a laparoscopy in patients with an uneventful medical history and normal pelvic examination therefore depends on how certain one wishes to be in finding fertility-impairing factors, and on the value attributed to treating Stages I and II endometriosis. Furthermore, it remains difficult

to establish the precise position and timing of laparoscopy in subfertile women due to a lack of randomized controlled studies (Bosteels *et al.*, 2007).
Practically, in couples with an otherwise unexplained subfertility, a negative CAT and a normal or inconclusive HSG, laparoscopy may be substituted by several cycles of combined gonadotrophins and intrauterine insemination, and subsequently IVF in those who failed to achieve a pregnancy (Fatum *et al.*, 2002). This strategy limits the number of unnecessary laparoscopies and does not introduce a large delay in treatment in the small proportion of women who would have been diagnosed with tubal pathology at laparoscopy, especially now that IVF is rapidly replacing reconstructive microsurgery as the treatment of choice in these patients. On the basis of our data, the clinical consequences of omitting laparoscopy in these low-risk patients seem limited, because after appropriate screening, little residual unexpected (and treatable) pathology is found. In CAT-positive women, laparoscopy remains justified in order to prevent unnecessary delay before going for IVF in those with abnormal findings and in order to prevent going for IVF prematurely in those in whom less demanding treatment options might still offer acceptable pregnancy chances. Furthermore, the subgroup of women with bilateral tubal occlusion at HSG also clearly benefits from laparoscopy with tubal testing, because laparoscopy shows not more than unilateral tubal pathology in the majority of these women (Mol *et al.*, 1999, Bosteels *et al.*, 2007), as was confirmed in our study. In this subgroup, immediate IVF can thus be avoided in the majority of women by excluding bilateral tubal occlusion at laparoscopy.

SUMMARY AND CONCLUSION

We evaluated three screening strategies, comprising serological testing (CAT and hs-CRP) and HSG, for assessing the risk of tubal pathology [i.e. reduced or (nearly) absent spontaneous pregnancy chances] in a population of subfertile women. Laparoscopy was used as a reference standard for detecting tubal pathology, and the occurrence of a spontaneous pregnancy was used as a surrogate marker for the absence of tubal pathology. Despite several limitations of the study, the results of the present study may be used to optimize the risk assessment for tubal factor subfertility. CAT is the most valuable test to screen for tubal pathology, because it accurately discerns low-risk patients (negative CAT) from high-risk patients (positive CAT). Based on biological grounds, hs-CRP may be applied in CAT-positive patients only. To assess the additional role of hs-CRP more accurately, further studies are needed. HSG has little additional value in estimating the risk of tubal pathology in low-risk and high-risk patients. In low-risk patients, HSG may be performed to confirm the absence of tubal pathology and to allow the patient to benefit from the positive perturbation effect of tubal flushing with oil-soluble contrast medium on pregnancy

rates. In high-risk patients, HSG is relatively contra-indicated owing to the 10% risk of post-HSG febrile and infectious morbidity in patients in whom tubal pathology is confirmed. In these high-risk patients and in women with bilateral tubal occlusion at HSG, laparoscopy is justified to provide a definite diagnosis. The role of laparoscopy in low-risk patients deserves critical reconsideration. The disadvantage of omitting laparoscopy would be that non-*C. trachomatis*-associated tubal pathology such as endometriosis (which was present in half of the women in our study population undergoing laparoscopy) remains undiagnosed and untreated. However, at least 90% of women with endometriosis at laparoscopy were classified as having Stages I or stage II disease, of which the lasting benefit of treatment is still a matter of debate. The consequences of omitting laparoscopy in these women may be of limited clinical significance, especially since eventually all those failing to conceive will be referred for IVF.

On the basis of the findings of the present observational study, we propose CAT as the first screening test for tubal pathology in subfertile women. In CAT-negative women, HSG may be performed because of its high specificity and, when using oil-soluble contrast medium, its fertility-enhancing effect. In CAT-positive women, hs-CRP is promising, although more studies are needed to further corroborate the value of hs-CRP in this respect. HSG has no additional diagnostic value and is relatively contra-indicated in CAT-positive women. The precise position and timing of laparoscopy deserves critical reappraisal and depends on the risk of tubal pathology, as assessed by screening tests. In CAT-negative patients and in patients with normal or inconclusive HSGs, laparoscopy may be omitted. In CAT-positive women and in women with abnormal HSGs, laparoscopy remains justified.

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Chapter 5

Toll-like receptor 4 in *Chlamydia trachomatis* infections: knockout mice, STD patients and women with tubal factor subfertility

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ABSTRACT

Chlamydia (*C.*) *trachomatis* is the most prevalent sexually transmitted bacterium in the world with almost 100 million new cases each year, some of which will develop tubal pathology. Clear differences in the clinical course of infections have been observed, and recently it has been shown that 40% is based on host genetic factors. We used an integrated approach based on infection of *toll-like receptor* (*TLR) 4* knockout mice and immunogenetic analysis of female sexually transmitted disease (STD) patients (susceptibility) and women with *C. trachomatis*-associated tubal factor subfertility (severity). The results in *TLR4* knockout mice suggest that the protection against reinfection is more solid in normal as compared to the *TLR4*-deficient mice. In humans, the functional *TLR4* single nucleotide polymorphism (SNP) studied was not involved in the susceptibility to infection. However, *C. trachomatis* immunoglobulin (Ig) G-positive subfertile women with tubal pathology were more than twice as likely to be carriers of the mutant *TLR4* +896 G allele as compared to those without tubal pathology; however, this observation did not reach statistical significance. In conclusion, both the murine model and the human immunogenetics studies show a slight effect upon *TLR4* deficiency in the severity of infection but not in the susceptibility to infection.

INTRODUCTION

Although *Chlamydia* (*C.*) *trachomatis* infection is a predominant cause of tubal pathology in subfertile women (Anestad *et al.*, 1997), not all women develop this complication following infection. The susceptibility, course and outcome of infectious diseases are determined by environmental factors (e.g. co-infection), bacterial factors (e.g. virulence) and host factors (e.g. immunogenetic differences between individuals).

C. trachomatis bacterial factors have been studied in relation to the clinical course of infection, such as symptomatic versus asymptomatic infection, lower versus upper genital tract infection, and clearance versus persistence. In previous studies, no strong associations were found between the different serovars of *C. trachomatis* and the clinical course (Ito *et al.*, 1990, Persson *et al.*, 1993, Dean *et al.*, 1995, Lampe *et al.*, 1995, Stothard *et al.*, 1998, Dean *et al.*, 2000, Morré *et al.*, 2000, Geisler *et al.*, 2003, Molano *et al.*, 2005). Host immune factors are considered more important determinants of the interpatient variability in the susceptibility, course and outcome of infectious diseases in general (Jeremias *et al.*, 1999, Smirnova *et al.*, 2003, Franchimont *et al*., 2004, Peeters *et al.*, 2004, Frodsham, 2005, Puthothu *et al.*, 2006), and *C. trachomatis* infections in particular (Kinnunen *et al.*, 2002). Recently, pattern recognition receptors (PRRs) of the toll-like receptor (TLR) family have been shown to be essential components of the host innate immune system, by playing a role in the recognition of pathogens and initiation of the immune response. TLR4 recognizes chlamydial lipopolysaccharide (LPS) via its co-receptor cluster of differentiation (CD) 14 (Poltorak *et al.*, 1998, Ohashi *et al.*, 2000). CD14 exists in a membrane-bound type (mCD14) and a soluble type (sCD14). Both mCD14 and sCD14 are able to form a complex with LPS and the LPS-binding protein (LBP). Combined with TLR4, this complex induces the nuclear factor kappa B (NF-κB) signal transduction cascade. Its end product NF-κB initiates the innate immune response by binding to specific DNA sequences in the nucleus, thereby enhancing the production of proinflammatory cytokines and acute phase proteins.

To investigate the role of specific genes and the proteins they encode, such as PRRs, in the susceptibility, course and outcome of diseases, different strategies can be employed. Two well-defined methods are the knockout (KO) mouse model and the human candidate gene approach.

The KO mouse model offers the opportunity to remove entire genes of interest from the genome, in order to compare the course and outcome (e.g. duration, amount of shedding and upper genital tract progression) of *C. trachomatis* infection and reinfection between KO mice and control mice, which possess the gene of interest. Previously, we developed a murine model using a human genital isolate of *C. trachomatis* in interferon (IFN)-γ and IFN-γ receptor KO mice, in which we were able

to demonstrate a major role of IFN-γ in controlling *C. trachomatis* infection (Ito and Lyons, 1999).
The human candidate gene approach, which is based on determining the frequencies of functional single nucleotide polymorphisms (SNPs) within phenotypically defined groups, can be used to investigate the relevance of genes in the susceptibility, course and outcome of diseases. SNPs in genes encoding for PRRs may influence receptor function, thereby leading to an aberrant immune response and an increased risk of adverse outcome of the disease. The *TLR4* +896 A>G SNP has been associated with hyporesponsiveness to LPS (Arbour *et al.* 2000). In a previous study, we did not observe an association between the *TLR4* +897 A>G SNP and tubal factor subfertility, but the cohort was relatively small (Morré *et al.*, 2003).
For the present study, the first objective was to assess the role of TLR4 in C3H/HeJ (*TLR4* deficient) and C3H/HeN (*TLR4* functional) mice. Secondly, we enlarged the cohort of subfertile women that was previously described (Morré *et al.*, 2003) and added a cohort of patients visiting a sexually transmitted diseases (STD) clinic with and without *C. trachomatis* infections to investigate the role of the *TLR4* +896 A>G SNP in the susceptibility to *C. trachomatis* infections and in the development of *C. trachomatis*-associated tubal factor subfertility.

MATERIALS AND METHODS

Murine model

Mice
Seven-week-old female C3H/HeJ (*TLR4* deficient) and C3H/HeN (*TLR4* functional) mice were purchased from Jackson Laboratories (USA) and allowed to acclimate for 2 weeks prior to use. Mice were maintained in accordance with American Association of Accreditation of Laboratory Animal Care guidelines, and were provided food and water ad libitum in an environmentally controlled BL-2 containment room with a 12-hour light/dark cycle.

C. trachomatis
A type pure and *Mycoplasma* free strain of *C. trachomatis* serovar D was propagated, titrated and isolated in cycloheximide-treated McCoy cell monolayers using standard techniques. Density gradient concentrated stock cultures were suspended in transport media and frozen at –70 °C until used.

Genital tract infection
In order to induce prolonged diestrous state and thus enhance the infection rate, progesterone in the form of medroxyprogesterone acetate (Depo-Provera®, Phar-

macia & Upjohn Co., USA) was administered subcutaneously in 2.5 mg doses, 10 and 3 days prior to infection. The mice were inoculated intravaginally with 10 µL of a *C. trachomatis* elementary body suspension containing 1 x 10^5 inclusion forming units. Control mice for the re-infection arm of the experiment received 10 µL of sterile transport media and were treated in every way similar to infected mice. All mice were progesterone-treated and infected with *C. trachomatis* in a similar manner 8 weeks later. All procedures were performed under protocols approved by the City of Hope National Medical Center and Beckman Research Institute Research Animal Care Committee.

Assessment of infection
The presence of *C. trachomatis* in the lower genital tract was determined by culturing material obtained by swabbing the vaginal vault and ectocervix every 2–7 days with a Dacron tipped urethral swab that was stored in transport media at –70°C until tested. Specimens were plated onto McCoy cell monolayers in duplicate 96-well plates, centrifuged and incubated at 37°C for 72 h. One plate was fixed, stained with iodine and enumerated for iodine straining inclusions, while the other plate was stored at –70°C and used to verify the status of primary culture negative specimens. An animal was considered productively infected if culture was positive on at least 1 day post-infection, and a specimen was considered positive if inclusions were observed in either the primary or secondary cultures. At the end of the experiment, all mice were visually examined for hydrosalpinx or other gross upper genital tract pathology.

Human study population

STD cohort
Women of Dutch Caucasian origin (n = 614), under the age of 33 (range 14—33 years), visiting the STD outpatient clinic in Amsterdam, the Netherlands, in the period of July 2001 to December 2004 participated in this cohort. Participants were asked to sign an informed consent form and fill out a questionnaire regarding their complaints at that moment, varying from increased discharge, having bloody discharge during and/or after intercourse, recent abdominal pain (not gastrointestinal or menses related) and/or dysuria. A cervical swab was taken for the detection of *C. trachomatis* DNA by polymerase chain reaction (PCR) (Van Doornum *et al.*, 2001). Peripheral venous blood was collected for the analysis of IgG antibodies against *C. trachomatis*. A titer of ≥ 1:50 was considered positive. Samples with grey-zone values, e.g. cut-off $\pm$ 10%, were repeated and considered positive when the result was positive, or again within the grey zone.

Subfertile women

The human study was performed in women who visited the Maastricht University Medical Centre between December 1990 and November 2000 because of subfertility. In all patients, blood was drawn at their initial visit for a *C. trachomatis* immunoglobulin (Ig) G antibody test (CAT). All spare sera were cryopreserved. Only patients who had undergone a laparoscopy and tubal testing as part of their fertility work-up were included in the present study. Since the prevalence of SNPs may depend on ethnic background, only Dutch Caucasian women were included. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded.

Two independent investigators, who were unaware of the CAT results, scored 259 successive laparoscopy reports to assess the presence of tubal pathology. Tubal pathology was defined as extensive peri-adnexal adhesions and/or distal occlusion of at least one tube (Land et al., 1998). In case of disagreement, consensus was reached by consultation. Of the 259 women who underwent a laparoscopy, only women who had tubal pathology (according to the above mentioned definition) or had no tubal pathology (no peri-adnexal adhesions and patent tubes) were included in the present study. Women who had minor or non-*C. trachomatis*-related abnormalities (any peri-adnexal adhesions and/or proximal occlusion of at least one tube) were excluded.

In the Netherlands, for retrospective analysis of anonymized patient data and stored sera, no ethical committee approval is required. In the fertility clinic of the Maastricht University Medical Centre, all couples are informed at intake about possible use of their anonymized data and stored sera for research purposes, and a "no objection procedure" is followed. Only patients who did not object participated in the present study.

C. trachomatis IgG antibody testing

IgG antibodies to *C. trachomatis* were detected using the species-specific *Chlamydia pneumoniae* IgG micro-immunofluorescence (MIF) test (AniLabsystems, Finland), as described previously (Den Hartog *et al.*, 2004). This species-specific test is able to detect IgG antibodies to both *C. pneumoniae* and *C. trachomatis* (using an antigen derived from a *C. trachomatis* lymphogranuloma venereum [LGV] strain, serovar L2). We previously studied the test performances of five commercially available *C. trachomatis* IgG tests, including the *C. trachomatis* IgG spot in the *C. pneumoniae* MIF (AniLabsystems, Finland) (Land *et al.*, 2003). In our hands, the *C. trachomatis* IgG titer obtained by the *C. pneumoniae* MIF (AniLabsystems, Finland) had the best predictive value for tubal factor subfertility (Land *et al.*, 2003). Therefore, we used this test in the present study. The cut-off titer used for a positive test was 32.

Immunogenetic analysis

For the immunogenetic analyses, genomic DNA was extracted from the cryopreserved serum samples using either the MagNaPure LC isolator according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany) or the High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany). Both techniques provide enough DNA for reproducible genetic analyses.

TLR4 +896 A>G gene polymorphism
Genotyping of the *TLR4* +896 A>G SNP (rs4986790) was performed with forward primer 5'-TTT ACC CTT TCA ATA GTC ACA CTC A-3' and reverse primer 5'-AGC ATA CTT AGA CTA CCT CCA TG-3'. PCR for restriction fragment length polymorphism (RFLP) analyses was performed on a thermal cycler GeneAmp 9700 (Perkin-Elmer Cetus, USA). PCR conditions were as follows: initial denaturation at 94˚C for 5 min, followed by 35 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for 30 s. The cycling was followed by a final extension step at 72˚C for 5 min, followed by cooling to 4˚C. The 102 base pairs (bp) amplicons were digested overnight at 37˚C with NcoI (New England Biolabs, UK) resulting in amplicons that either were cut in two fragments of 80 bp and 22 bp (G allele) or were not restricted (A allele). These fragments were analyzed by electrophoresis on 4% low melting agarose gels (Tebu-Bio, the Netherlands) stained with ethidium bromide.

Control group for background genotyping
For ethnic-matched background genotyping, genomic DNA was extracted from whole blood of 166 healthy Dutch Caucasian employees of the VU University Medical Center. They gave written informed consent for use of their anonymized sera to serve as control sera for genetic research purposes.

Statistical analyses

The genotype distribution was tested for Hardy-Weinberg equilibrium to assess Mendelian inheritance. Fisher's exact or chi-square tests were used to compare the genotypes between *C. trachomatis* IgG-positive and IgG-negative subfertile women with and without tubal pathology and the healthy control group. $P < 0.05$ was considered statistically significant.

RESULTS

Murine model

The median duration of infection between the previously non-infected control groups of each strain were similar (Table I). This was similar to what was observed during the initial infection arm of the experiment. In the first week, the amount of *C. trachomatis* shedding in the control groups was slightly higher in the wild-type mice as compared to the *TLR4*-deficient mice. Upon re-infection, a significant level of protection was observed in both mouse strains when compared to the initial infection in the appropriate age- and conditions-matched control group: C3H/HeJ (*TLR4* deficient), 31.5 vs. 7 days; and C3H/HeN (wild-type), 26 days vs. 4 days. Remarkably, none of the wild-type mice was infected 4 days after re-infection, while 75% of the *TLR4*-deficient mice were still *C. trachomatis* positive after 1 week, and one mouse even during the complete follow-up period of 2 weeks.

Table I: Duration of genital tract infection and culture results in C3H/HeJ (*TLR4* deficient) and C3H/HeN (*TLR4* functional) mice following *C. trachomatis* infection or mock-infection (at t = 0) and *C. trachomatis* infection (at t = 56).

	Time	t = 0			t = 56			t = 58	t = 60	t = 63	t = 66	t = 70
	Mouse	Infection	Infection duration	Median duration	Infection	Infection duration	Median duration	Culture results on indicated day (IFU)				
C3H/HeJ (*TLR4* deficient)	C1	CT	28	26	CT	7	7	6,300	1,850	360	0	0
	C2	CT	24		CT	7		26,870	1,190	400	0	0
	C3	CT	35		CT	4		17,580	10	0	0	0
	C4	CT	24		CT	21		3,180	150	680	660	250
	J1	Mock	-	-	CT	28	31.5	33,900	5,370	12,900	1,680	1,390
	J2	Mock	-		CT	14		13,000	8,900	4,220	70	280
	J5	Mock	-		CT	35		28,810	14,040	21,150	8,200	2,950
	J6	Mock	-		CT	42		24,230	5,600	23,400	4,160	1,670
C3H/HeN (*TLR4* functional)	B1	CT	17	28	CT	4	4	68,120	2,770	0	0	0
	B2	CT	35		CT	4		35,010	20	0	0	0
	B3	CT	21		CT	4		6,240	+	0	0	0
	B25	CT	35		CT	4		33,450	100	0	0	0
	I1	Mock	-	-	CT	24	26	72,800	4,970	8,720	830	1,120
	I2	Mock	-		CT	24		23,400	13,520	10,460	120	110
	I4	Mock	-		CT	28		18,510	30,160	48,360	2,310	290
	I5	Mock	-		CT	35		22,360	67,600	10,920	3,350	530

Abbreviations: CT = *C. trachomatis* serovar D; IFU = inclusion-forming units; mock = infection with sterile transport media; *TLR4* = toll-like receptor 4.

However, and although small, there is a suggestion that the protection was more solid in C3H/HeN (*TLR4* wildtype) compared to C3H/HeJ (*TLR4* deficient) mice. Finally, at the conclusion of the experiment there was no hydrosalpinx or other gross upper tract pathology observed in any mice of either strain.

Human candidate gene approach

Of the 259 women who underwent laparoscopy, 227 participated in the present study. Of these, 43 (19%) had tubal pathology and 184 (81%) did not. Thirty-two women had minor or non-*C. trachomatis*-related abnormalities and were excluded. *C. trachomatis* IgG antibodies were present in 39 women, of whom 26 (67%) had tubal pathology and 13 (33%) did not have tubal pathology. *C. trachomatis* IgG antibodies were absent in 188 women, of whom 17 (9%) had tubal pathology and 171 (91%) did not have tubal pathology.

Susceptibility of infection

The genotype distribution for the STD cohort in shown in Table II and was in Hardy-Weinberg equilibrium showing Mendelian inheritance. The women who were positive versus those who were negative for *C. trachomatis* DNA had an equal genotype distribution, as did the more extreme subgroups of *C. trachomatis* DNA-positive and *C. trachomatis* serology-positive women versus the *C. trachomatis* DNA-negative and *C. trachomatis* serology-negative women (Table II).

Table II. *TLR4* genotype distribution in female STD patients.

TLR4 +896 A>G	n	1.1 (AA)		1.2 (AG)		2.2 (GG)	
		n	%	n	%	n	%
Total STD patients	614	530	86.3	83	13.5	1	0.2
DNA+	193	169	87.6	24	12.4	0	0.0
DNA+, IgG+	140	121	86.4	19	13.6	0	0.0
DNA–	421	361	85.7	59	14.0	1	0.2
DNA–, IgG–	331	283	85.5	47	14.2	1	0.3

Abbreviations: IgG = immunoglobulin G; TLR4 = toll-like receptor 4; STD = sexually transmitted disease.

Severity of infection

The genotype distribution was in Hardy-Weinberg equilibrium in the subfertile women and the ethnic-matched control group. The overall genotype distribution in the cohort of subfertile women was 88% AA, 12% AG and 0% GG. The genotype distribution in subfertile women with tubal pathology (86% AA, 14% AG, 0% GG) did not differ from the distribution in subfertile women without tubal pathology (89%

AA, 11% AG, 0% GG). Introduction of *C. trachomatis* IgG serology, with special attention to *C. trachomatis* IgG-positive subfertile women with and without tubal pathology, did alter the observed genotype distribution, and a clear trend (OR 2.9) was noticed towards a higher frequency of *TLR4* +896 A>G SNP carriage in *C. trachomatis* IgG-positive subfertile women with tubal pathology (19% *G) as compared to those without tubal pathology (8% *G). The results are summarized in Table III.

Table III. TLR4 genotype distribution in women with subfertility.

TLR4 +896 A>G	n	1.1 (AA)		1.2 (AG)		2.2 (GG)	
		n	%	n	%	n	%
All subfertile women	227	200	88.1	27	11.9	0	0.0
TP+	43	37	86.0	6	14.0	0	0.0
TP –	184	163	88.6	21	11.4	0	0.0
CT + TP+	26	21	80.8	5	19.2	0	0.0
CT + TP –	13	12	92.3	1	7.7	0	0.0
Control group	166	148	89.2	17	10.2	1	0.6

Abbreviations: CT = C. trachomatis IgG; TLR4 = toll-like receptor 4; TP = tubal pathology. Control group = ethnic-matched healthy employees of the VU University Medical Center.

DISCUSSION

The current study used an integrated approach to study the role of *TLR*4 in the susceptibility to and severity of *C. trachomatis* infection based on both the murine model and human candidate gene approaches in women. The results of the KO mouse model indicate an important level of protection against *C. trachomatis* re-infection, as reflected by a faster clearance, as compared to mice that were infected only once. Our data suggest that the protection is more solid in *TLR4* functional mice as compared to the *TLR4*-deficient mice. *C. trachomatis* IgG-positive subfertile women with tubal pathology were more than twice as likely to be carriers of the mutant *TLR4* +896 G allele compared to those without tubal pathology. This difference, however, was not statistically significant due to sample size limitations.

Although the *TLR4*-deficient mice had clear differences in the level of shedding and duration of infection following re-infection, the role was minimal and as anticipated. It has previously been shown that TLR2, but not TLR4, is more essential for recognition of a different chlamydia species (*C. pneumoniae*) (Prebeck *et al.*, 2001), LPS-signaling (Erridge *et al.*, 2004), and the development of murine tubal pathology (Darville *et al.*, 2003), respectively. Our results are consistent with these findings, indicating that TLR4 does play only a modest role in the recognition of *C. trachomatis* and that signaling through TLR2 may be a more prominent element in the immune response. Indeed Karimi *et al*. (2009) have recently shown that specific *TLR2*

haplotypes were associated with the development of tubal pathology and the severity of infection.
The current lack of a strong association between the *TLR4* SNP studied and *C. trachomatis*-associated tubal factor subfertility may be explained by several other factors besides the suggested prominent role of TLR2, rather than TLR4, in the immune response to *C. trachomatis*. First, Erridge and co-workers (2003) have shown that only the rare homozygous carriers of the *TLR4* +897 A>G SNP are less responsive to LPS, whereas heterozygous carriage does not affect LPS responsiveness. Our study cohort consisted of women with a normal *TLR4* genotype or heterozygous *TLR4* +897 A>G SNP carriers, whereas no patients were homozygous SNP carriers. However, LPS is not the only ligand for TLR4 and both human heat shock protein 60 (hHSP60) and chlamydia HSP60 (cHSP80) are also ligands for TLR4. Secondly, the recognition of *C. trachomatis* involves a complex system of multiple PRRs. Partial or complete loss of receptor function may be compensated by other receptors, preserving an adequate immune response via other pathways. Deregulation of the immune response, leading to a more severe course of *C. trachomatis* infection, may only occur when different pathways are affected concomitantly, i.e. when multiple SNPs in multiple PRR genes (in a so-called carrier trait) are present. This hypothesis has already been tested for several diseases (Smirnova *et al.*, 2003, Hugot *et al.*, 2001, El-Omar *et al.*, 2003, Machado *et al.*, 2003, Heresbach *et al.*, 2004), and a preliminary study has shown promising results for *C. trachomatis*-associated tubal pathology (Den Hartog *et al.*, 2006).
In the current study, the most prominent effect was seen between *C. trachomatis*-positive women with and without serologically confirmed *C. trachomatis* infection. We added cHSP60 serology to the assessment (data not shown), since TLR4 also responds to cHSP60, and increased expression of cHSP60 has been reported in infertile women (Jha *et al.*, 2009). Interestingly, all women positive for *C. trachomatis* IgG and cHSP60 IgG, and with the mutant G allele in *TLR4*, had tubal pathology. This potentially interesting phenomenon will be further explored in larger cohorts, and by adding SNPs in other *TLRs* and genes involved in the recognition and inflammatory processes upon infection. This is of importance, since not all women with tubal pathology are positive for *C. trachomatis* IgG and cHSP60 IgG and yet have the G allele in TLR4.

CONCLUSIONS

TLR4 functional mice seem to be more protected against *C. trachomatis* re-infection as compared to the *TLR4* deficient mice. In humans, the single *TLR4* +896 A>G SNP does not play a major role in susceptibility, as shown in women with uncomplicated *C. trachomatis* infection. In women with serological responses to *C. trachomatis* and

carrying the *TLR4* SNP, the risk of developing tubal pathology was higher but did not reach statistical significance. Since other receptors may compensate for the loss of function in patients carrying the *TLR4* SNP, further studies are necessary to determine if carrying multiple SNPs in multiple PRR genes and having serological responses to cHSP60 has a more profound impact on the development of tubal pathology following *C. trachomatis* infection as compared to the risk associated with carrying a single SNP in a single gene.

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Chapter 6

The *CD14* functional gene polymorphism –260 C>T is not involved in either the susceptibility to *Chlamydia trachomatis* infection or the development of tubal pathology

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ABSTRACT

Background
The functional polymorphism -260 C>T in the LPS-sensing TLR4 co-receptor *CD14* gene enhances the transcriptional activity and results in a higher CD14 receptor density. Individuals carrying the T/T genotype also have significantly higher serum levels of soluble CD14. The T allele of this polymorphism has recently been linked to *Chlamydia* (*C.*) *pneumoniae* infection. We investigated the role of the *CD14* -260 C>T polymorphism in the susceptibility to and severity (defined as subfertility and/or tubal pathology) of *C. trachomatis* infection in Dutch Caucasian women.

Methods
The different *CD14* -260 C>T genotypes were assessed by PCR-based RFLP analysis in three cohorts: 1) A cohort (n = 576) of women attending a STD clinic, 2) a cohort (n = 253) of women with subfertility, and 3) an ethnically matched control cohort (n = 170). The following variables were used in the analysis: In cohort 1, the *C. trachomatis* DNA status, *C. trachomatis* IgG serology status and self-reported symptoms, and in cohort 2, the *C. trachomatis* IgG serology status and the tubal status at laparoscopy.

Results
In the control cohort, the CC, CT and TT genotype distribution was 28.2%, 48.2%, and 23.5%, respectively. No differences were found in the overall prevalence of *CD14* -260 genotypes (28.1%, 50.7%, and 21.2%) in cohort 1 when compared to the control cohort. Also no differences were observed in women with or without *C. trachomatis* DNA, with or without serological *C. trachomatis* responses, with or without symptoms, or in combinations of these three variables. In subfertile women with tubal pathology (cohort 2, n = 50), the genotype distribution was 28.0%, 48.0%, and 24.0%, and in subfertile women without tubal pathology (n = 203), 27.6%, 49.3% and 23.2%. The genotype distribution was unchanged when *C. trachomatis* IgG status was introduced in the analyses.

Conclusion
The *CD14* -260 C>T genotype distributions were identical in all three cohorts, showing that this polymorphism is not involved in the susceptibility to or severity of sequelae of *C. trachomatis* infection.

INTRODUCTION

Chlamydia species are related to a broad clinical spectrum of human disease, including *Chlamydia* (*C.*) *pneumoniae* in lung and cardiovascular disease, *C. psittaci* in pulmonary emphysema and psittacosis, and *C. trachomatis* in ocular and urogenital infections (Smieja *et al.*, 2002a, Smieja *et al.*, 2002b, Numazaki *et al.*, 2003).
C. trachomatis is the most prevalent sexually transmitted disease in Europe and the USA. Due to the mostly asymptomatic course of infection, these women will most likely not be treated, resulting in an enhanced risk for the development of late complications, which include pelvic inflammatory disease, ectopic pregnancy and tubal infertility.
The female reproductive tract is a very complex system where many factors, including hormones, vaginal flora and immune mediators, combine to provide protection on the one hand, while on the other hand maintaining an environment suitable for conception (Wira *et al.*, 2005). Clear differences in the clinical course of infection have been described and are due to an interaction between environmental (e.g. co-infection), bacterial (e.g. virulence factors) and host factors (genetic differences between individuals). In previous studies, no clear associations have been demonstrated between *C. trachomatis* serotype, *C. trachomatis* genotype, and the course of *C. trachomatis* infection (Workowski *et al.*, 1994, Lyons *et al.*, 2004), although differences in cytotoxicity for different serovars have been described (Lyons *et al.*, 2005) and an association between *C. trachomatis* serovar G and cervical squamous cell carcinoma has been suggested (Anttila *et al.*, 2001). In addition, virulence gene expression studies and genomic comparisons of strains, isolated from clearly symptomatic or asymptomatic infected persons, revealed no strong role for the *C. trachomatis* bacterium in relation to the course of infection (Morré *et al.*, 2000, Pannekoek *et al.*, 2005).
A limited number of studies has recently demonstrated the influence of host genetic factors on the susceptibility to and the severity of *C. trachomatis* infection. Host factors including HLA-DQ and interleukin 10 (IL-10) have been associated with chlamydia infection (Kinnunen *et al.*, 2002).
The toll-like receptor (TLR) family is a group of pattern recognition receptors, which recognize several microbial products, including bacterial cell wall components and DNA (Takeda *et al.*, 2003). Poltorak *et al.* (1998) associated TLR4 with lipopolysaccharide (LPS) recognition in mice. Further studies in mice corroborated these data (Beutler, 2000, Netea *et al.*, 2002a), while studies in humans demonstrated associations between *TLR4* mutations and LPS hyporesponsiveness (Arbour *et al.*, 2000). We did not observe an association between the *TLR4* Asp299Gly polymorphism in patients with tubal pathology, although the study population was relatively small (Morré *et al.*, 2003). The lack of association can be explained by recent publications showing that heterozygous carriage of the *TLR4* Asp299Gly mutation does not affect

LPS responsiveness, and that only the rare homozygous carriers are less responsive to LPS (Erridge *et al.*, 2003).
Cluster of differentiation (CD) 14 acts as a co-receptor for TLR4 and confers responsiveness to LPS, a component of the cell wall of most Gram-negative bacteria. CD14 forms a complex with LPS and the LPS-binding protein (LBP) (Figure I) (Wright *et al.*, 1990). Combined with TLR4, this complex induces nuclear factor (NF)-κB associated immune responses including the release of a broad spectrum of cytokines that include tumor necrosis factor alpha (TNF-α), IL-1, IL-6, and IL-8 to initiate immune response (Baldini *et al.*, 1999).

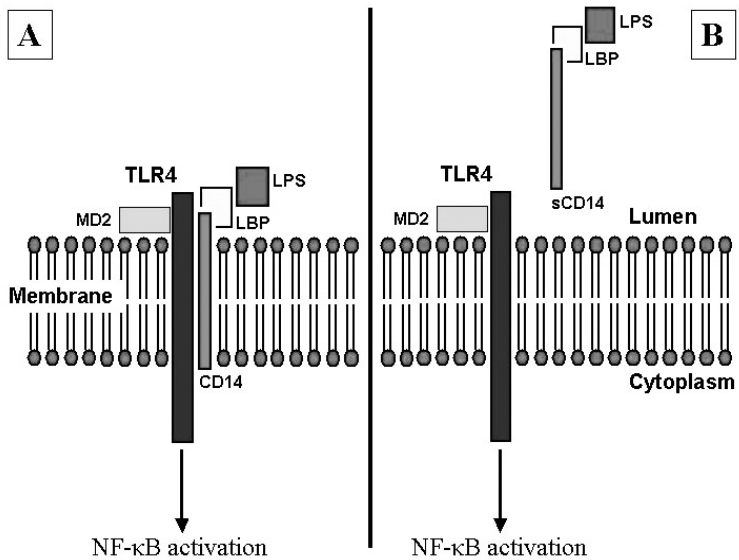

Figure I. CD14 localization**.** A: Membrane-bound CD14 (mCD14) complexed with TLR4 and the LBP-LPS complex. Panel B: Soluble CD14 (sCD14).
Abbreviations: CD = cluster of differentiation; LPB = LPS-binding protein; LPS = lipopolysaccharide; MD2 = myeloid differentiation protein-2; NF-κB = nuclear factor-κB; TLR = toll-like receptor.

The promotor region of the *CD14* gene contains a single nucleotide polymorphism (SNP) at position -260. The -260 C>T genetic variation affects the binding of transcription factors (Zhang *et al.*, 1994), and has been associated with levels of soluble CD 14 (sCD14) and inversely associated with serum IgE levels (Baldini *et al.*, 1999). This SNP has been associated with myocardial infarction (Hubacek *et al.*, 1999), Crohn's disease (Klein *et al.*, 2003) and an increased susceptibility to develop chronic spondyloarthropathy in women (Repo *et al.*, 2002).
Eng *et al.* (2004) demonstrated that carriers of the T allele of this promotor polymorphism have a higher expression of both membrane-bound CD14 (mCD14) and sCD14, and that TNF-α production is increased in the homozygous *CD14* -260 T car-

riers when stimulated with either *C. pneumoniae* or *C. trachomatis*. In a recent article, Rupp *et al.* (2004) described an association between the mutant allele and an increased susceptibility to chronic *C. pneumoniae* infection in coronary artery disease patients. Since the *CD14* -260 C>T is functional (Eng *et al.*, 2004) and is associated with *C. pneumoniae* infection (Rupp *et al.*, 2004), one could hypothesize that in *C. trachomatis* infection this polymorphism could influence the susceptibility to and severity of this most prevalent sexually transmitted bacterium which is associated with female infertility.
Therefore, we investigated the role of the *CD14* -260 C>T polymorphism in the susceptibility to and severity (defined as subfertility and/or tubal pathology) of *C. trachomatis* infection in Dutch Caucasian women. A cohort of women attending a Sexually Transmitted Diseases (STD) outpatient clinic was used to assess the susceptibility to *C. trachomatis* infection, taking into account both *C. trachomatis* DNA and *C. trachomatis* IgG detection, symptoms and co-infections. A cohort of subfertile women with or without clinically well-defined tubal pathology was used to assess the role of CD14 in the severity of sequelae of *C. trachomatis* infection.

METHODS

Patient populations

STD cohort
Women of Dutch Caucasian origin (n = 576), under the age of 33 (range 14 to 33 years; median 22 years) and visiting the STD outpatient clinic in Amsterdam, the Netherlands, were included in this study (collection period: July 2001—December 2004) (Table I). All 576 women were consecutively included as the first part of a large prospective study. For every *C. trachomatis* DNA positive woman, two consecutive *C. trachomatis* DNA negative controls were included in the study. The women were asked to sign an informed consent form and to fill out a questionnaire, regarding their complaints at that moment, varying from increased discharge, having bloody discharge during and/or after intercourse, recent abdominal pain (not gastrointestinal or menses related) and/or dysuria. A cervical swab was taken for the detection of *C. trachomatis* DNA by polymerase chain reaction (PCR; COBAS AMPLICOR, Hoffman—La Roche, Basel, Switzerland) (Van Doornum *et al.*, 2002).

Table I. Patient characteristics in the STD and subfertility cohorts.

		STD cohort (n = 576)	Subfertility cohort (n = 253)
CT DNA	+	184	NA
	-	392	NA
CT IgG (ELISA)[a]	+	217	NA
	-	359	NA
CT IgG (MIF)[b]	+	NA	41
	-	NA	212
Co-infections	+	175	NA
Candida albicans		160	NA
Neisseria gonorrhoea		7	NA
Trichomonas vaginalis		6	NA
Herpes simplex virus 1		2	NA
Herpes simplex virus 2		5	NA
	-	401	NA
Symptoms	+	221	NA
Vulvovaginal discharge		157	NA
Abdominal pain		81	NA
Dysuria		58	NA
Bleeding during/after coitus		25	NA
	-	335	NA
Age	Average	23.6 y	30 y
	Range	15—41 y	19—39 y
	Median	23 y	31 y
Tubal pathology	+	NA	50
	-	NA	203

[a] *C. trachomatis* IgG ELISA (Medac, Germany); ≥ 1:50= positive; [b] *C. trachomatis* IgG MIF (AniLabSystems, Finland); ≥ 32 = positive. Abbreviations: CT = *Chlamydia trachomatis*; IgG = immunoglobulin G; NA = not available; STD cohort = female visitors of a Sexually Transmitted Diseases outpatient clinic; subfertility cohort = subfertile women undergoing a fertility work-up.

Peripheral venous blood was collected for the analysis of IgG antibodies against *C. trachomatis* (ELISA; 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 the micro-organisms *Candida albicans, Neisseria gonorrhoea, Trichomonas vaginalis* and *Herpes simplex virus 1/2* may result in symptoms similar to *C. trachomatis* infection. Infection status for these micro-organisms was recorded. *Herpes simplex virus 1/2* was detected according to the methods described by Bruisten *et al.* (2001). *Neisseria gonorrhoea* was detected according to methods described by Spaargaren *et al.* (2001). *Trichomonas vaginalis* was cultured on Trichosel medium according to standard procedures (Murray *et al.*, 1995), and

detection of *Trichomonas vaginalis* was according the methods described by Van der Schee *et al.* (2001). *Candida albicans* was cultured on Chrom agar, and detection of *Candida albicans* was performed according to standard procedures (Murray *et al.*, 1995).

Subfertility cohort
The study was performed in 253 consecutive Dutch Caucasian women who visited the department of Obstetrics and Gynaecology of the Academisch Ziekenhuis Maastricht, the Netherlands, between December 1990 and November 2000 because of subfertility (Land *et al.*, 2003). In these women, a laparoscopy with tubal testing had been performed as part of their fertility work-up. Preoperatively, blood was drawn from all patients for *C. trachomatis* 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 (Land *et al.*, 1998). Subfertile women who had no peri-adnexal adhesions and had patent tubes at laparoscopy served as negative controls. Based on these criteria, 50 women had tubal pathology and 203 women served as controls.
IgG antibodies to *C. trachomatis* were detected with a species-specific MIF test (AniLabSystems, Finland), as described previously (Land *et al.*, 2003), with comparable sensitivity and specificity as compared to the IgG ELISA from Medac used for the STD cohort (Morré *et al.*, 2002). A positive *C. trachomatis* IgG MIF test was defined as a titre ≥ 1:32. Findings at laparoscopy were correlated with the MIF test results. Based on the MIF test, 41 women were found to be *C. trachomatis* IgG-positive, while 212 were *C. trachomatis* IgG-negative. Of the *C. trachomatis* IgG-positive women, 28 (68.8%) had tubal pathology, while 22 women (10.4%) of the *C. trachomatis* IgG-negative women had tubal pathology.

Healthy controls
A healthy Dutch Caucasian control group (n = 170) was included to assess the general frequency of the *CD14* -260 genotypes in the Dutch Caucasian population.

DNA extraction

STD cohort
Eukaryotic DNA from peripheral blood mononuclear cells (PBMCs) was isolated using the isopropanol isolation method. In short: 100 µL PBMC in phosphate-buffered saline 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 min at 65°C and left to cool at room temperature. 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 according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Healthy controls
Blood was collected in EDTA-tubes and stored at room temperature until the genomic DNA was extracted from PBMCs according to an in-house DNAzol (Invitrogen, the Netherlands) isolation procedure.

Immunogenetic analysis

CD14 -260 C>T gene polymorphism
The C>T substitution in the proximal *CD14* promoter GC box at position -260 from the translation start site (NCBI SNP CLUSTER ID:rs2569190) results in a *Hae*III restriction site. We developed a PCR assay using the primers 5' TCA CCT CCC CAC CTC TCT T 3' (sense) and 5' CCT GCA GAA TCC TTC CTG TT 3' (antisense) (Invitrogen Life Technologies, Breda, the Netherlands), flanking this restriction site. Amplification was performed using a thermal cycler Perkin-Elmer 9700 (Applied Biosystems, Forter City, CA, USA). The parameters were an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 1 min. The final elongation was at 72°C for 7 min, followed by a cooling to 4°C. The 107 base pairs (bp) fragments were digested overnight at 37°C with *Hae*III (Invitrogen, the Netherlands) resulting in fragments that either were cut in two fragments of 83 bp and 24 bp (allele C) or were not restricted (T allele). These fragments were analyzed by electrophoresis on 4% low melting agarose gels (Tebu-Bio, the Netherlands) stained with ethidium bromide.

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). Fisher exact and χ^2 tests were used to test

for differences in *CD14* allele/genotype/carrier frequencies between the (sub)groups. *P*-values < 0.05 were considered statistically significant.

RESULTS

All genotype distributions assessed were in Hardy-Weinberg equilibrium. The *CD14* -260 C>T SNP was assessed in the STD, subfertility and control cohorts.

CD14 -260 in the susceptibility to *C. trachomatis* infection

To determine the effects of *CD14* -260 C>T on the susceptibility to *C. trachomatis* infection, the prevalence of *CD14* -260 C>T genotypes were assessed in the STD cohort (Table II). The overall genotype distribution was 28.1% CC, 50.7% CT and 21.2% TT. This distribution was comparable to the healthy controls (Figure II). The distribution was 28.8% CC, 50.0% CT and 21.2% TT in *C. trachomatis* DNA-positive women, while in *C. trachomatis* DNA-negative women the distribution was 27.8% CC, 51.0% CT and 21.2% TT. In women with or without serological *C. trachomatis* responses, the distribution was 30.4% CC, 49.3% CT and 20.3% TT, and 26.7% CC, 51.5% CT and 21.7% TT, respectively. No differences could be observed in women with or without symptoms. Co-infection with other micro-organisms or combinations of these four variables (*C. trachomatis* DNA, *C. trachomatis* serology, symptoms and micro-organisms) did not introduce statistically significant differences or trends in *CD14* genotype distributions.

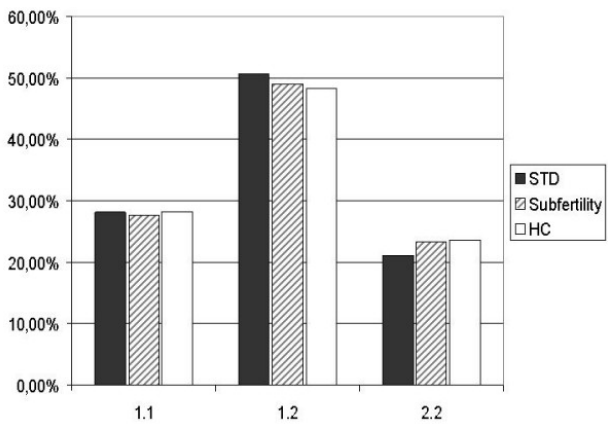

Figure II. *CD14* genotype distribution in the STD, subfertility and control cohorts.
Abbreviations: HC = healthy controls; STD cohort = female visitors of a Sexually Transmitted Diseases outpatient clinic; subfertility cohort = subfertile women undergoing a fertility work-up.

Table II. *CD14* genotype distribution in the STD cohort.

			CD14 -260 C>T					
			1.1 (CC)		1.2 (CT)		2.2 (TT)	
		Total	n	%	n	%	n	%
CT IgG+	Total	217	66	30.4%	107	49.3%	44	20.3%
	CT DNA+	135	38	28.1%	69	51.1%	28	20.7%
	Co-infection+	42	12	28.6%	24	57.1%	6	14.3%
	Symptoms+	56	14	25.0%	31	55.4%	11	19.6%
	Lower abd. pain+	17	4	23.5%	10	58.8%	3	17.6%
	CT DNA-	82	28	34.1%	38	46.3%	16	19.5%
	Co-infection+	29	12	41.4%	11	37.9%	6	20.7%
	Symptoms+	43	16	37.2%	14	32.6%	13	30.2%
	Lower abd. pain+	17	5	29.4%	8	47.1%	4	23.5%
CT IgG-	Total	359	96	26.7%	185	51.5%	78	21.7%
	CT DNA+	49	15	30.6%	23	46.9%	11	22.4%
	Co-infection+	16	3	18.8%	10	62.5%	3	18.8%
	Symptoms+	19	5	26.3%	11	57.9%	3	15.8%
	Lower abd. pain+	10	3	30.0%	6	60.0%	1	10.0%
	CT DNA-	310	81	26.1%	162	52.3%	67	21.6%
	Co-infection+	88	20	22.7%	53	60.2%	15	17.0%
	Symptoms+	103	26	25.2%	51	49.5%	26	25.2%
	Lower abd. pain+	37	6	16.2%	20	54.1%	11	29.7%
Healthy controls		170	48	28.2%	82	48.2%	40	23.5%

Distribution of the *CD14* genotype in *C. trachomatis* IgG-positive and IgG-negative female visitors of a Sexually Transmitted Diseases outpatient clinic, divided in CT DNA-positive and CT DNA-negative patients, and subdivided in patients with co-infection with other micro-organisms (*Neisseria gonorrhoea*, *Trichomonas vaginalis*, *Candida albicans*, *Herpes simplex virus 1 & 2*), symptoms (vulvovaginal discharge, abdominal pain, dysuria, bleeding during/after coitus) and lower abdominal pain. Abbreviation: CT = *C. trachomatis*.

CD14 -260 in the severity of sequelae of *C. trachomatis* infection

The effect of *CD14* -260 C>T on the severity of sequelae of *C. trachomatis* infection was assessed in a cohort of subfertile women with clinically well-defined tubal pathology. The overall genotype distribution in the cohort was 27.7% CC, 49.0% CT and 23.3% TT (Figure II). The genotype distribution in women with tubal pathology was similar to the distribution in women without tubal pathology (28.0% CC, 48.0% CT and 24.0% TT, and 27.6% CC, 49.3% CT and 23.2% TT respectively) and to the distribution in the healthy controls (Table III). Introduction of *C. trachomatis* IgG serology, with special attention to *C. trachomatis* IgG-positive women who did develop tubal pathology as compared to those who did not develop tubal pathology, did not alter the observed genotype distribution.

Table III. *CD14* genotype in the subfertility cohort.

		CD14 -260 C>T					
		1.1 (CC)		1.2 (CT)		2.2 (TT)	
Total	253	70	27.7%	124	49.0%	59	23.3%
TP+	50	14	28.0%	24	48.0%	12	24.0%
TP-	203	56	27.6%	100	49.3%	47	23.2%
CT IgG+ and TP+	28	9	32.1%	15	53.6%	4	14.3%
CT IgG+ and TP-	13	4	30.8%	6	46.2%	3	23.1%
Healthy controls	170	48	28.2%	82	48.2%	40	23.5%

Distribution of the *CD14* genotype in *C. trachomatis* IgG-positive and IgG-negative subfertile women undergoing a fertility work-up, divided in women with and without tubal pathology, and in *C. trachomatis* IgG positive women with and without tubal pathology. Abbreviations: CT = *C. trachomatis*; IgG = immunoglobulin G; TP = tubal pathology.

DISCUSSION

We did not find an association between the functional up-regulating *CD14* -260 C>T polymorphism and the susceptibility to or subsequent severity of sequelae of *C. trachomatis* infection, as assessed in the STD and subfertility populations (Figure II). However, these results do not exclude that a still unknown CD14 expression decreasing SNP may influence the course of *C. trachomatis* infection.

Recent studies have shown that chlamydia LPS is capable of inducing an inflammatory response through CD14 (Ingalls *et al.*, 1995, Heine *et al.*, 2003), although the potency to induce an inflammatory response was 100—1 000 times less when compared to the responses induced by *S. minnesota*, *N. gonorrhoea* (Ingals *et al.*, 1995) and the enterobacteria *S. enterica* and *E. coli* (Heine *et al.*, 2003). Heine *et al.* (2003) demonstrated that the CD14-associated inflammatory response was TLR4 but not TLR2 mediated. These results are corroborated by studies showing the role of the CD14-TLR4-MD2 complex in intracellular signalling by LPS (Poltorak *et al.*, 1998, Akashi *et al.*, 2003) and studies showing the dependency on CD14 of phagocytosis of Gram-negative bacteria (Grunwald *et al.*, 1996).

The absence of an association between CD14 and susceptibility to *C. trachomatis* infection might be explained by the compartmentalization of TLR4. The differential expression of TLR4 has been described in immortalized cell-lines derived from the female urogenital tract (Fichorova *et al.*, 2002), and has recently been demonstrated in cells isolated from patients by Pioli *et al.* (2004) and Fazeli *et al.* (2005). TLRs 1—6 were found to be expressed in the epithelia of the female urogenital tract. TLR2 and TLR4 were the only toll-like receptors with a clear differential expression, low expression in the lower urogenital tract and high expression in the upper genital tract (Pioli *et al.*, 2004, Fazeli *et al.*, 2005). The expression remained similar in all subjects irrespective of age or status of the reproductive cycle (Fazeli *et*

al., 2005). It is hypothesized that through this expression pattern, TLR4 modulates immunological tolerance in the lower genital tract and induces host defence against ascending infection in the upper genital tract (Fazeli *et al.*, 2005). In the upper genital tract, Fazeli *et al.* (2005) found TLR4 positive vacuole-like structures that seemed to be secreted from endocervical glands. A secretory form of TLR4 has been described in mice, where the soluble TLR4 appears to inhibit LPS-mediated signals, while at the same time sTLR4 mRNA is upregulated by LPS (Iwami *et al.*, 2000). This may represent a feedback mechanism to prevent excessive responses to LPS in the endocervix, which can be seen as a boundary between the lower and upper genital tract. Further evidence for the regulation of immune responses to LPS by TLR4 is provided by the study of Harju *et al.* (2005), who demonstrated the intrauterine expression of TLR4 and endotoxin responsiveness in mice in the perinatal period. mCD14 is expressed on human endometrial stromal cells, but not on endometrial epithelial gland cells. The epithelial cells are dependent on sCD14 for LPS recognition (Bäckhed *et al.*, 2002). sCD14 is present in the cervical mucosa and may be present in the endometrium (Lawn *et al.*, 2000).

Combining the afore-mentioned studies with the knowledge that CD14 can signal through TLR4, it might be hypothesized that the absence of an association between the *CD14* -260 SNP and the susceptibility to *C. trachomatis* infection might be due to the low expression or absence of TLR4 in the lower urogenital tract. In the upper genital tract, strict regulation of immune responses to LPS by TLR4 may inhibit CD14 signalling through TLR4 (Iwami *et al.*, 2000, Harju *et al.*, 2005), thus limiting the influence of CD14 on the development of tubal pathology.

However, this hypothesis does not take into account the ability of CD14 to signal through TLR2 (Netea *et al.*, 2002b). Furthermore, it has been demonstrated that non-LPS components of *C. pneumoniae* can stimulate cytokine production through TLR2-dependent but CD14-independent pathways (Yoshioka *et al.*, 2001). If a similar mechanism would exist for *C. trachomatis*-induced cytokine production in urogenital tract infections, the role of CD14 may be questioned.

Since TLR2 is involved in chlamydia-induced transforming growth factor β, an anti-inflammatory cytokine with an important role in fibrosis, and thus very likely in post-infection tubal pathology, it might explain why CD14 polymorphisms may not severely impact the development of tubal pathology (Yoshioka *et al.*, 2001).

Darville *et al.* (2003) have demonstrated that TLR2 is an important mediator of innate immune responses in *C. trachomatis* infection in mice and plays an important role in early production of immune mediators and development of tubal pathology (Darville *et al.*, 2003, Erridge *et al.*, 2004). In a recent publication by Opitz *et al.* (2005), it was shown that *C. pneumoniae* is capable of activating endothelial cells by TLR2 as initial extracellular *C. pneumoniae* receptor, whereas NOD1 was shown to be a potent intracellular immune receptor for *C. pneumoniae* in endothelial cells. Further research may extend these results to *C. trachomatis* infections. Overall, the

recognition of bacterial LPS involves a complex system of multiple receptors and a complex orchestration of protein-protein interactions (Triantafilou and Triantafilou, 2002).

CONCLUSION

Our study showed that the functional up-regulating *CD14* -260 C>T SNP did neither influence the susceptibility to nor the severity of late sequelae of *C. trachomatis* infection. However, this does not exclude a prominent role for CD14 in the course of an active *C. trachomatis* infection and not yet described CD14 expression decreasing SNPs may affect the course of *C. trachomatis* infection profoundly. Further studies on the immunogenetics of *C. trachomatis* infection will provide more insight in the clear differences in the clinical course that this microorganism induces in individuals and lead to potential vaccine candidates.

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Chapter 7

Do host genetic traits in the bacterial sensing system play a role in the development of *Chlamydia trachomatis*-associated tubal pathology in subfertile women?

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ABSTRACT

Background
In women, *Chlamydia (C.) trachomatis* upper genital tract infection can cause distal tubal damage and occlusion, increasing the risk of tubal factor subfertility and ectopic pregnancy. Variations, like single nucleotide polymorphisms (SNPs), in immunologically important host genes are assumed to play a role in the course and outcome of a *C. trachomatis* infection. We studied whether genetic carrier traits (carrying multiple SNPs in different genes) in the bacterial sensing system are associated with an aberrant immune response and subsequently with tubal pathology following a *C. trachomatis* infection. The genes studied all encode for pattern recognition receptors (PRRs) involved in sensing bacterial components.

Methods
Of 227 subfertile women, serum was available for *C. trachomatis* IgG antibody testing and genotyping (common versus rare allele) of the PRR genes *TLR9*, *TLR4*, *CD14* and *CARD15/NOD2*. In all women, a laparoscopy was performed 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.

Results
Following a *C. trachomatis* infection (i.e. *C. trachomatis* IgG positive), subfertile women carrying two or more SNPs in *C. trachomatis* PRR genes were at increased risk of tubal pathology compared to women carrying less than two SNPs (73% vs 33% risk). The differences were not statistically significant (P = 0.15), but a trend was observed.

Conclusion
Carrying multiple SNPs in *C. trachomatis* PRR genes tends to result in an aberrant immune response and a higher risk of tubal pathology following *C. trachomatis* infection. Larger studies are needed to confirm our preliminary findings.

INTRODUCTION

A large variation exists in the individual response to a *Chlamydia* (*C.*) *trachomatis* infection. Some women clear a *C. trachomatis* infection adequately without developing tissue damage, whereas others get a persistent infection which may ascend to the upper genital tract, increasing the risk of tubal damage and subfertility. The susceptibility, course and outcome of infectious diseases are determined by environmental factors, virulence factors of the pathogen and host factors.

Immunogenetic studies evaluate the role of genetic variations in immunologically important host genes as determinants of the susceptibility, course and outcome of infectious diseases. Among these variations are single nucleotide polymorphisms (SNPs), in which one nucleotide has been substituted, inserted or deleted. This may lead to synthesis of a potentially aberrant protein, or to up- or downregulation of the normal protein, and subsequently to an aberrant immune response, increasing the risk of late sequelae of infectious diseases (e.g. tubal pathology following a *C. trachomatis* infection).

In the present study, we have evaluated SNPs in genes encoding for pattern recognition receptors (PRRs). PRRs are present on or in circulating cells of the innate immune system (e.g. macrophages) and local cells (e.g. epithelial cells of the upper genital tract). PRRs are involved in the bacterial sensing pathways of the innate immune system by recognizing the so-called pathogen-associated molecular patterns (PAMPs), which are pathogen-specific cell wall components or intracellular components. Since different PRRs recognize different PAMPs, pathogen recognition and initiation of the immune response is a complex and flexible system.

Carrying a SNP in a single PRR may not result in a large effect on disease severity, since other PRRs may compensate for the partial loss of function in a specific pathogen recognition route. Subsequently, SNPs in only one PRR may not play a significant role as risk factors for the development of *C. trachomatis*-associated tubal pathology, as shown for the PRR toll-like receptor (TLR) 4 (Morré *et al.*, 2003) and its co-receptor cluster of differentiation (CD) 14 (Ouburg *et al.*, 2005). However, carrying multiple SNPs in one gene or in multiple genes (in so-called carrier traits) may be associated with an increased risk of tubal pathology. Smirnova *et al*. (2003) have found that combinations of *TLR4* variants are markedly more common in patients with meningococcal infections, whereas single variants are not over-represented in those patients. In studies on gastrointestinal malignancies, it has been concluded that carrying multiple pro-inflammatory polymorphisms is associated with an increased risk of gastric cancer (El-Omar *et al.*, 2003, Machado *et al.*, 2003). Furthermore, studies on the relationship between caspase recruitment domain (CARD) 15/nucleotide oligomerisation domain (NOD) 2 genetic variants, of which SNP8, SNP12 and SNP13 are most studied, and Crohn's disease have shown that compound heterozygous subjects (carriers of two different genetic variants, e.g. SNP12

genotype 1.2 and SNP13 genotype 1.2) have a higher risk of Crohn's disease as compared to homozygous subjects (carriers of the same genetic variant on both chromosomes, e.g. SNP12 genotype 2.2) (Hugot *et al.*, 2001, Heresbach *et al.*, 2004). Analogous to these findings, we hypothesized that carrying multiple genetic variations in multiple PRRs (in a so-called carrier trait) may increase the risk of *C. trachomatis*-associated tubal pathology in subfertile women. According to their biological function (recognition of *C. trachomatis* PAMPs: see Table I), four PRRs were selected: TLR9, TLR4, CD14 and CARD15/NOD2. Five relatively common SNPs, which are assumed to influence the receptor function, in these four PRR genes were studied in this carrier trait analysis (see Table I).

Table I. The pattern recognition receptors (PRRs), which recognize *C. trachomatis* pathogen-associated molecular patterns (PAMPs), and the single nucleotide polymorphisms (SNPs) studied.

PRR	PAMP	SNP
TLR9	CpG-rich motifs	-1237 T>C and +2848 G>A
TLR4	LPS and HSP	+896 A>G
CD14	LPS and HSP (co-receptor of TLR4)	-260 C>T
CARD15/NOD2	Peptidoglycans	Leu1007fsinsC (SNP13)

Abbreviations: A = adenine; C = cytosine; CARD = caspase recruitment domain; CD = cluster of differentiation; CpG = cytosine-phosphate-guanine; fsins = frameshift insertion; G = guanine; HSP = heat shock protein; Leu = leucine-rich repeat domain; LPS = lipopolysaccharide; NOD = nucleotide oligomerisation domain; T = thymine; TLR = toll-like receptor.

METHODS

Study population

The study was performed in women who visited the Academic Hospital Maastricht between December 1990 and November 2000 because of subfertility. In all patients, blood was drawn at their initial visit for a Chlamydia IgG antibody test (CAT). All spare sera were cryopreserved. Only patients who had undergone a laparoscopy and tubal testing as part of their fertility work-up were included in the present study. Since the prevalence of SNPs may depend on ethnical background, only Dutch Caucasian women were included. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded. In the Netherlands, for retrospective analysis of anonymized patient data and stored sera no ethical committee approval is required. In the fertility clinic of the Academic Hospital Maastricht, all couples are informed at intake about possible use of their anonymized data and stored sera for research purposes, and a "no objection procedure" is followed. Only patients having not objected participated in the present study.

Two independent investigators, who were unaware of the CAT results, scored 259 successive 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 (Land *et al.*, 1998). In case of disagreement, consensus was reached by consultation.
Of the 259 women who underwent a laparoscopy, 43 (17%) had tubal pathology (according to the above-mentioned definition) and 184 (71%) had no tubal pathology (no peri-adnexal adhesions and patent tubes), and these 227 women participated in the present study. Thirty-two women (12%) had minor or non-*C. trachomatis*-related abnormalities (any peri-adnexal adhesions and/or proximal occlusion of at least one tube) and were excluded.

C. trachomatis IgG antibody testing

IgG antibodies to *C. trachomatis* were detected using the species-specific *Chlamydia pneumoniae* IgG micro-immunofluorescence (MIF) test (AniLabsystems, Finland), as described previously (Den Hartog *et al.*, 2004). This species-specific test is able to detect IgG antibodies to both *C. pneumoniae* and *C. trachomatis* (using an antigen derived from a *C. trachomatis* LGV strain, serovar L2). We have previously studied the test performances of five commercially available *C. trachomatis* IgG tests, including the *C. trachomatis* IgG spot in the *C. pneumoniae* MIF (AniLabsystems, Finland) (Land *et al.*, 2003). In our hands, the *C. trachomatis* IgG titre obtained by the *C. pneumoniae* MIF (AniLabsystems, Finland) had the best predictive value for tubal factor subfertility (Land *et al.*, 2003). Therefore, we have used this test in the present study. The cut-off titre used for a positive test was 32.

Immunogenetic analysis

For the immunogenetic analyses, genomic DNA was extracted from the cryopreserved serum samples using either the MagNaPure LC isolator according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany) or the High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany). Both techniques provide enough DNA for reproducible genetic analyses. Genotyping was performed using a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP)-assay or TaqMan-assay as described previously (Morré *et al.*, 2002, Murillo *et al.*, 2002, Lammers *et al.*, 2005, Ouburg *et al.*, 2005). The SNPs studied are summarized in Table I. Chromosomal locations and further information on the genes studied are: *TLR9* chromosomal location 3p21.3, *TLR9* -1237 T>C rs5743836 and *TLR9* +2848 G>A rs352140; *TLR4* chromosomal location 9q32-q33, *TLR4* +896 A>G rs4986790; *CD14* chromosomal location 5q31.1, *CD14* -260 C>T rs25691909; *CARD15/NOD2* chromo-

somal location 16q21, *CARD15/NOD2* Leu1007fsinsC (SNP13) rs2066847. For ethnically matched background genotyping, genomic DNA was extracted from whole blood of 97 healthy Dutch Caucasian employees of the VU University Medical Center. They gave written informed consent for use of their anonymized sera to serve as control sera for genetic research purposes.

Statistical methods

The genotype distribution was tested for Hardy-Weinberg equilibrium to assess Mendelian inheritance. Fisher's exact or χ^2 tests were used to compare the single genotypes between *C. trachomatis* IgG-positive and IgG-negative subfertile women with and without tubal pathology and the healthy control group. Subsequently, the single genotypes were used to define carrier traits. The carrier traits were tested in χ^2 and trend analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Of all 227 subfertile women participating in the present study, 43 (19%) had tubal pathology, whereas 184 (81%) did not have tubal pathology. *C. trachomatis* IgG antibodies were present in 39 women, of whom 26 (67%) had tubal pathology and 13 (33%) did not have tubal pathology. *C. trachomatis* IgG antibodies were absent in 188 women, of whom 17 (9%) had tubal pathology and 171 (91%) did not have tubal pathology.

For all genes studied, the genotype distribution was in Hardy-Weinberg equilibrium in the subfertile women and the ethnically matched control group.

The genotype distribution did not differ between subfertile women, the *C. trachomatis* IgG-positive subgroup of subfertile women and the healthy control group (Table II), indicating that the subfertile women participating in the study reflect an average Dutch Caucasian population regarding the genotype distribution.

Table II. The risk of tubal pathology (TP) in relation to the genotype of the single genes studied.

		1.1		1.2 and 2.2	
		n	Risk of TP	n	Risk of TP
TLR9 -1237 T>C	All subfertile women	155 (68%)	20%	72 (32%)	17%
	CT+ subfertile women	26 (67%)	62%	13 (33%)	77%
	Control group	66 (68%)	-	31 (32%)	-
TLR9 +2848 G>A	All subfertile women	45 (20%)	18%	182 (80%)	19%
	CT+ subfertile women	6 (15%)	50%	33 (85%)	70%
	Control group	15 (15%)	-	82 (85%)	-
TLR4 +896 A>G[a]	All subfertile women	200 (88%)	19%	27 (12%)	22%
	CT+ subfertile women	33 (85%)	64%	6 (15%)	83%
	Control group	87 (90%)	-	10 (10%)	-
CD14 -260 C>T[b]	All subfertile women	60 (26%)	17%	167 (74%)	20%
	CT+ subfertile women	12 (31%)	67%	27 (69%)	67%
	Control group	26 (27%)	-	71 (73%)	-
CARD15/NOD2 Leu1007fsinsC (SNP13)	All subfertile women	211 (93%)	18%	16 (7%)	25%
	CT+ subfertile women	37 (95%)	65%	2 (5%)	100%
	Control group	95 (98%)	-	2 (2%)	-

All subfertile women: n = 227, of whom 19% has TP. CT + (*C. trachomatis* IgG-positive) subfertile women: n = 39, of whom 67% has TP. Control group: n = 97 ethnically matched healthy employees of the VU University Medical Center. 1.1 = normal genotype (homozygous for the common allele). 1.2 = heterozygous SNP carrier (one common allele and one rare allele). 2.2 = homozygous SNP carrier (homozygous for the rare allele). Abbreviations: see Table I. [a] Adapted from Morré *et al*. (2003). [b] Adapted from Ouburg *et al*. (2005).

Single gene analysis

The risk of tubal pathology was assessed in all subfertile women and in the *C. trachomatis* IgG-positive subgroup in relation to the genotype of *TLR9*, *TLR4*, *CD14* and *CARD15/NOD2* (Table II; Figure I). An increasing risk of tubal pathology was observed across the genotypes in all genes except *CD14*. Carrying SNPs in these genes increased the risk of tubal pathology (on average almost 20%). These differences did not reach statistical significance. These single genotypes were used to define carrier traits.

Carrier trait analysis

The SNPs in the single genes were combined in carrier traits. The risk of tubal pathology was assessed in *C. trachomatis* IgG-positive and IgG-negative subfertile women in relation to the number of SNPs. Carrying two or more SNPs did not influence the risk of tubal pathology in *C. trachomatis* IgG-negative women as compared to *C. trachomatis* IgG-negative women carrying less than two SNPs (9% vs. 8% risk respectively; Figure II). However, carrying multiple SNPs doubled the risk of tubal

pathology in *C. trachomatis* IgG-positive women as compared to *C. trachomatis* IgG-positive women with less than two SNPs (73% vs. 33% risk respectively; Figure II). These differences did not reach statistical significance (P = 0.15) but a clear trend was observed.

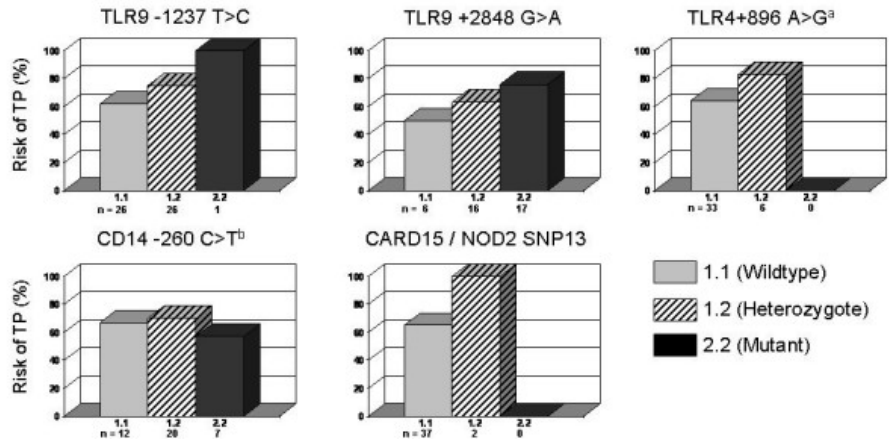

Figure I. The risk of tubal pathology (TP) in *C. trachomatis* IgG-positive subfertile women in relation to the genotype of the single pattern recognition receptor genes.
[a] Adapted from Morré *et al*. (2003). [b] Adapted from Ouburg *et al*. (2005). Abbreviations: see Table I.

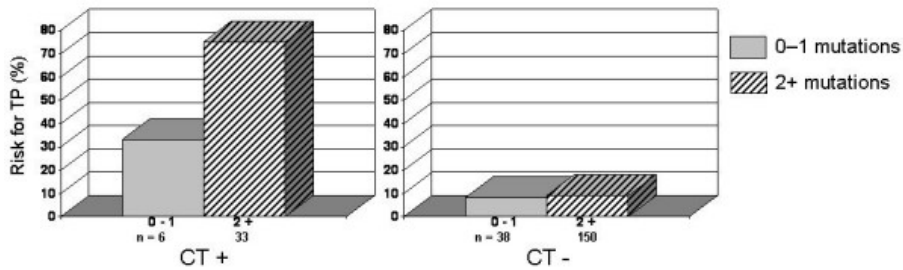

Figure II. The risk of tubal pathology (TP) in *C. trachomatis* IgG-positive (CT+) and IgG-negative (CT-) subfertile women in relation to carrying five single nucleotide polymorphisms in four pattern recognition receptor genes.

DISCUSSION

Over the last decade, immunogenetic studies have provided insight in the pathogenesis of and susceptibility to infectious diseases. So far, the role of SNPs in immunologically relevant genes has been established in numerous diseases, e.g. sexually transmitted infections (Jeremias *et al.*, 1999, Kinnunen *et al.*, 2002, Wang *et al.*, 2005) and inflammatory bowel diseases (Crohn's disease and ulcerative colitis) (Franchimont *et al.*, 2004, Peeters *et al.*, 2004). In this study, we analyzed the role of single SNPs and multiple SNPs in multiple genes (in a so-called carrier trait) as risk factors of *C. trachomatis*-related tubal pathology and we confirmed our hypothesis that a carrier trait based on genes in bacterial sensing pathways had a stronger association with the risk of tubal pathology than a single gene analysis.

Recent studies have shown the value of genetic traits in complex diseases. Carrying multiple SNPs in the same gene, or multiple SNPs in multiple genes, has been associated with an increased risk of infectious diseases and malignancies (Hugot *et al.*, 2001, El-Omar *et al.*, 2003, Smirnova *et al.*, 2003, Heresbach *et al.*, 2004). Analogous to these findings, we hypothesized that the disregulation of the immune response by the presence of multiple SNPs may lead to an even higher risk of tubal pathology following a *C. trachomatis* infection as compared to carrying a single SNP.

In this study, we investigated the role of five SNPs in four genes which are assumed to play a role in the recognition of *C. trachomatis* (see Table I). An adequate recognition of *C. trachomatis* by PRRs is the first step in the immune response. Recent studies have shown that TLR1-9 are expressed in the human female genital tract. TLR4 and its co-receptor CD14 are predominantly expressed in the fallopian tubes, where they may play an important role in the innate host defence mechanism against ascending *C. trachomatis* infections (Pioli *et al.*, 2004, Schaefer *et al.*, 2004, Fazeli *et al.*, 2005). Regarding CARD15/NOD2, it is not clear whether it plays a role in the *C. trachomatis* recognition in the genital tract, although NOD proteins have been shown to be involved in the intracellular sensing of *C. pneumoniae* in endothelial cells (Opitz *et al.*, 2005).

Our data show a doubling of the risk of tubal pathology in *C. trachomatis* IgG-positive women carrying two or more SNPs as compared to *C. trachomatis* IgG-positive women carrying less than two SNPs (73% vs. 33% risk). The differences did not reach statistical significance ($P = 0.15$) due to the small sample size (227 subfertile women in total, including 39 *C. trachomatis* IgG-positive subfertile women, of whom 67% has tubal pathology). If the association found in this pilot study would be confirmed in a larger cohort, a 50% increase in sample size (to 341 subfertile women) would result in a significant difference ($P = 0.047$) between *C. trachomatis* IgG-positive subfertile women carrying two or more SNPs versus *C. trachomatis* IgG-positive subfertile women carrying less than two SNPs regarding the risk of tubal pathology. A 100% increase in sample size (to 454 subfertile women) would result in

a strong association (*P* = 0.016; OR 5.3) between carrying two or more SNPs and an increased risk of tubal pathology. Increasing the sample size twofold would not be possible, however, in our setting in a reasonable time frame. Although the present study was performed in a large fertility clinic and patients were included during a ten-year period, the number of affected women remained small, due to the low prevalence of *C. trachomatis* IgG-positivity in combination with both carrying multiple SNPs and tubal pathology.

From our results it can be concluded that an adequate recognition of the pathogen at the site of infection seems to be a relevant step in the immune response, and that carrying multiple SNPs in multiple *C. trachomatis* PRR genes tends to increase the risk of an aberrant immune response and tubal pathology. To draw significant conclusions, our hypothesis should be retested in further studies using a larger cohort.

As expected, a difference in risk of tubal pathology between *C. trachomatis* IgG-positive women and *C. trachomatis* IgG-negative women was observed. In previous studies, the presence of *C. trachomatis* IgG antibodies, an indicator of a previous *C. trachomatis* infection, has been associated with tubal pathology (Punnonen *et al.*, 1979). Our data show that carrying multiple SNPs in bacterial sensing pathways and a previous *C. trachomatis* infection synergistically enhance the risk of tubal pathology, while carrying these SNPs does not influence the risk of tubal pathology in the absence of a previous *C. trachomatis* infection (Figure II).

CONCLUSION

We hypothesized that carrier traits (i.e. carrying multiple SNPs in multiple genes) that likely result in an aberrant immune response are associated with tubal pathology following a *C. trachomatis* infection. In 227 subfertile women, we studied five variations in four genes encoding for PRRs, which recognize several pathogen-associated molecular patterns of *C. trachomatis*. The presence of two or more SNPs tends to correlate with an increased risk of tubal pathology following a *C. trachomatis* infection as compared to a lower number of SNPs. Further studies in a larger cohort are needed to confirm our preliminary findings. An adequate recognition of *C. trachomatis* by receptors in the genital tract seems to be a relevant step in the immune response, and may play a role in protecting the host against the development of late sequelae following a *C. trachomatis* infection.

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Chapter 8

Detection of *Chlamydia trachomatis* in endometrium of subfertile women: a pilot study using immunohistochemical staining and PCR

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In preparation

ABSTRACT

Background
Chlamydia (*C.*) *trachomatis* infections are a known cause of tubal factor subfertility. Persistent *C. trachomatis* endometrial infection may also negatively influence fertility by decreasing the implantation capacity of the endometrium. The present study evaluates the detection rate of C. *trachomatis* in endometrial samples of subfertile women with and without tubal pathology using immunohistochemical staining and polymerase chain reaction (PCR).

Methods
Forty subfertile women were included in the present study. *C. trachomatis* antibody testing (CAT) and laparoscopy with tubal testing were performed as part of the fertility work-up. Endometrial samples were collected for immunohistochemical staining (with four different antibodies directed against *C. trachomatis* major outer membrane protein and heat shock protein) and PCR. *C. trachomatis*-infected human epithelial (HEp2) cells and mock-infected HEp2 cells served as positive and negative controls respectively.

Results
Of all forty women participating in the present study, 17 women (42.5%) had a positive CAT, and 9 women (22.5%) had tubal pathology at laparoscopy. For none of the four antibodies, significant differences were found in immunohistochemical staining results between endometrial samples of subfertile women with a positive or negative CAT, and with or without tubal pathology. All thirteen endometrial samples tested by PCR were negative.

Conclusions
No correlation was found between immunohistochemical staining results in endometrial samples and clinical parameters in our cohort of 40 patients. PCR results were negative in all thirteen samples tested, but this subgroup was very small. Larger cohorts are needed to re-evaluate the relation between CAT, *C. trachomatis* PCR on endometrial samples, tubal status and clinical outcome (implantation rates).

INTRODUCTION

Chlamydia (*C.*) *trachomatis* lower genital tract infections usually remain asymptomatic and untreated, and therefore may ascend from the cervix to the upper genital tract. It is generally accepted that long term persistence of *C. trachomatis* in the upper genital tract increases the risk for late sequelae, such as tubal pathology, causing ectopic pregnancy and subfertility.

Several test methods have been described to detect *C. trachomatis* in genital tract samples. Nucleic acid amplification tests (NAATs) on both DNA and RNA levels, such as polymerase chain reaction (PCR), have been shown to be the most accurate detection methods (sensitivity $\geq$ 90% and specificity 98-100%) (Van Dyck *et al*, 2001, Watson *et al.*, 2002). NAATs are capable of detecting DNA and RNA sequences with a length of less than 50 base pairs or nucleotides respectively (Noguchi *et al.*, 2002). Immunohistochemical staining methods have other targets (proteins or lipopolysaccharides) and are less accurate in detecting *C. trachomatis* as compared to NAATs because no amplification occurs and larger epitopes are needed to identify the micro-organism (Noguchi *et al.*, 2002).

Despite the known association between *C. trachomatis* genital tract infections and subfertility, active endocervical *C. trachomatis* infections are infrequently found in subfertile women because the interval between initial infection and subfertility is generally several years. A mean prevalence of ~ 3% has been demonstrated in studies using NAATs on cervical samples obtained from subfertile women (Witkin *et al.*, 1994, Witkin *et al.*, 1995, Eggert-Kruse *et al.*, 1997, Macmillan and Templeton, 1999, Land *et al.*, 2002, Debattista *et al.*, 2004, De Barbeyrac *et al.*, 2006, Guven *et al.*, 2007, Machado *et al.*, 2007, Imudia *et al.*, 2008, Svenstrup *et al.*, 2008, Wilkowska-Trojniel *et al.*, 2009, Dietrich *et al.*, 2010) (Table I).

When using NAATs on tubal samples of women with ectopic pregnancy or tubal factor subfertility, a mean prevalence of *C. trachomatis* of 12% has been found (Osser and Persson, 1992, Lan *et al.*, 1995, Haeusler *et al.*, 1997, Gérard *et al.*, 1998, Hinton *et al.*, 2000, Barlow *et al.*, 2001, Noguchi *et al.*, 2002, Debattista *et al.*, 2004, Bjartling *et al.*, 2007) (Table II). *C. trachomatis* detection rates in tubal tissue as high as 70% show that women who have previously undergone a *C. trachomatis* infection (usually during adolescence) and who suffer from late sequelae, may still harbour the micro-organism in the upper genital tract. Others, however, were unable to confirm the presence of *C. trachomatis* in tubal tissue of women with ectopic pregnancy (Osser and Persson, 1992, Bjartling *et al.*, 2007) (Table II). The large differences in prevalence figures between the studies mentioned may be explained by differences in study cohort, sample size, preservation method of the tissue (fresh-frozen or paraffin-embedded) or test method.

Table I. The prevalence of *C. trachomatis* in cervical samples of subfertile women using nucleic acid amplification tests.

Author and year	Patients	Controls	Results (% CT-positive)
Witkin *et al.*, 1994	198 subfertile women undergoing IVF	None	Overall: 9.1% (18/198) Pregnant: 4.4% (3/68) Not pregnant: 11.5% (15/130)
Witkin *et al.*, 1995	307 subfertile women undergoing IVF	None	Overall: 6.5% (20/307) Ongoing pregnancy: 1.8% (2/112) Abortion: 9.8% (4/41) Not pregnant: 9.6% (13/135) No fertilization: 5.3% (1/19)
Eggert-Kruse *et al.*, 1997	150 subfertile women undergoing a fertility work-up	None	1.3% (2/150)
Macmillan and Templeton, 1999	210 subfertile women undergoing a fertility work-up	None	1.9% (4/210)
Land *et al.*, 2002	255 subfertile women undergoing a fertility work-up	None	2.0% (5/255)
Debattista *et al.*, 2004	39 subfertile women undergoing a laparoscopy	None	0% (0/39)
De Barbeyrac *et al.*, 2006	261 subfertile women undergoing IVF	None	2.7% (7/261)
Guven *et al.*, 2007	31 unexplained subfertile women	31 women undergoing sterilization	Patients: 3.2% (1/31) Controls: 0% (0/31)
Machado *et al.*, 2007	33 women with tubal factor subfertility 22 women with $\geq$ 1 prior ectopic pregnancy	55 parous women	Tubal factor subfertility: 6.1% (2/33) Prior ectopic pregnancy: 0% (0/22) Controls: 0% (0/55)
Imudia *et al.*, 2008	236 subfertile women undergoing a fertility work-up	None	2.2% (5/231)
Svenstrup *et al.*, 2008	212 subfertile women undergoing a fertility work-up	None	0.5% (1/212)
Wilkowska-Trojniel *et al.*, 2009	71 subfertile women 23 women with tubal factor subfertility 48 women with non-tubal factor subfertility	None	Overall: 8.5% (6/71) Tubal factor subfertility: 8.7% (2/23) Non-tubal factor subfertility: 8.3% (4/48)
Dietrich *et al.*, 2010	202 subfertile women undergoing a laparoscopy	None	1.0% (2/202)

Several studies have evaluated the involvement of the endometrium (i.e. plasma cell endometritis and, more specific, endometrial *C. trachomatis* infection) in women with *C. trachomatis* lower or upper genital tract infection. Histopathological manifestations of endometritis (plasma cells and infiltrations of leucocytes) have been found in 30-40% of women with *C. trachomatis* cervicitis (Paavonen *et al*., 1985a; Wiesenfeld *et al*., 2002) and in 70% of women with suspected pelvic inflammatory disease (Paavonen *et al*., 1985b). The micro-organism itself has been detected in the endometrium of 17% (range 3-50%) of women with signs of endometritis or tubal pathology in studies using NAATs (Winkler *et al.*, 1984, Lan *et al.*,

1995, Stern *et al.*, 1996, Barlow *et al.*, 2001) (Table III). In studies using immunohistochemical staining methods, *C. trachomatis* prevalence figures of 4-57% have been reported in women with ectopic pregnancy or chronic endometritis (Winkler *et al.*, 1984, Toth *et al.*, 2000).

Table II. The prevalence of *C. trachomatis* in tubal samples of women with subfertility and/or tubal pathology using nucleic acid amplification tests.

Author and year	Patients	Controls	Results (% CT-positive)
Osser and Persson, 1992	86 women with ectopic pregnancy	None	0% (0/86)
Lan *et al.*, 1995	37 women with ectopic pregnancy	None	2.7% (1/37)
Haeusler *et al.*, 1997	20 subfertile women undergoing a hysteroscopy + laparoscopy	None	5.0% (1/20)
Gérard *et al.*, 1998	10 women with ectopic pregnancy	None	70.0% (7/10)
Hinton *et al.*, 2000	77 women with chronic salpingitis	77 women with histologically normal tubes	Patients: 11.7% (9/77) Controls: 0% (0/77)
Barlow *et al*., 2001	30 women with ectopic pregnancy 14 women with tubal factor subfertility	50 women undergoing hysterectomy or sterilization	Ectopic pregnancy: 46.7% (14/30) Tubal factor subfertility: 35.7% (5/14) Controls: 6.0% (3/50)
Noguchi *et al.*, 2002	11 women with ectopic pregnancy, pyosalpinx or hydrosalpinx	None	36.4% (4/11)
Debattista *et al.*, 2004	41 subfertile women undergoing a laparoscopy	None	2.4% (1/41)
Bjartling *et al.*, 2007	55 women with ectopic pregnancy	None	0% (0/55)

Table III. The prevalence of *C. trachomatis* in endometrial samples of women with endometritis or tubal pathology using nucleic acid amplification tests.

Author	Patients	Controls	Results (% CT-positive)
Lan *et al*., 1995	18 women with ectopic pregnancy	None	16.7% (3/18)
Stern *et al*., 1996	38 women with abnormal bleeding and chronic endometritis	None	2.6% (1/38)
Barlow *et al*., 2001	30 women with ectopic pregnancy 14 women with tubal factor subfertility	50 women undergoing hysterectomy or sterilization	Ectopic pregnancy: 20.0% (6/30) Tubal factor subfertility: 50.0% (7/14) Controls: 2.0% (1/50)

It has been hypothesized that persistent endometrial infections (as reflected by the presence of microbial products or inflammatory mediators) affect either the conceptus or the implantation capacity of the endometrium, leading to implantation failure (Kamiyama *et al.*, 2004, Romero *et al.*, 2004). More specifically, ongoing *C. trachomatis* infections have been associated with lower implantation rates and lower pregnancy rates in women undergoing IVF (Witkin *et al.*, 1994, Witkin *et al.*, 1995, Pacchiarotti *et al.*, 2009).
However, as mentioned before, detection rates of *C. trachomatis* in endometrial samples as described in literature vary between 3 and 57%, due to — amongst others — different test methods and different study populations. Therefore, the precise clinical significance and consequences of harbouring *C. trachomatis* in the endometrium remain unclear. The aim of the present pilot study was to evaluate the detection rate of C. *trachomatis* in endometrial samples of subfertile women with and without tubal factor subfertility using two different test methods: immunohistochemical staining (detecting *C. trachomatis* major outer membrane protein [MOMP] and heat shock protein [HSP]) and PCR (detecting *C. trachomatis* DNA).

MATERIALS AND METHODS

Study population

The study population consisted of 40 women who visited the Maastricht University Medical Center Fertility Clinic and who underwent a laparoscopy with tubal testing as part of the fertility work-up between April 2001 and February 2005. In all patients blood was drawn at their initial visit for the purpose of *C. trachomatis* IgG antibody testing (CAT). In patients with a negative CAT and an otherwise normal basic fertility evaluation, tubal status was evaluated initially by hysterosalpingography (HSG). If HSG showed abnormalities, or if patients did not conceive in the six months following HSG, the fertility evaluation was concluded by laparoscopy with tubal testing using methylene blue dye. In patients with a positive CAT no HSG was performed, but tubal status was evaluated primarily by laparoscopy. Patients with a history of pelvic surgery (except for an uneventful appendectomy or Caesarean section) or pelvic inflammatory disease, and patients with suspected severe endometriosis, based on history and findings at pelvic examination, also underwent laparoscopy directly.
All women received a single oral dose of azithromycin 1000 mg on the evening preceding laparoscopy. Just prior to starting laparoscopy, endometrial samples were collected by transvaginal biopsy using a sampling device (Gynotec, Malden, the Netherlands). The obtained tissue was embedded in paraffin. All patients gave their

written informed consent for collection and use of the endometrial samples for the purpose of fertility research.
Tubal pathology was defined as extensive peri-adnexal adhesions and/or at least unilateral tubal occlusion at laparoscopy.

Serological methods

During the years, different CAT tests have been used in our Fertility Clinic. In serum samples obtained before 2003, the micro-immunofluorescence (MIF) test by Biomérieux (Boxtel, the Netherlands) was performed, whereas in 2003 it was replaced by the MIF test of AniLabsystems (Vantaa, Finland), which by then had been shown to be superior (Land *et al.*, 2003). Both MIF tests were performed according to the manufacturer's instructions (Land *et al.*, 2003). The cut-off level for a positive test was 32 for both MIF tests.

Patient characteristics

Of all 40 subfertile women participating in the present study, 80% had a primary subfertility and 20% had a secondary subfertility. The mean age at intake was 30.9 years (range 25-38). The mean duration of subfertility was 23 months (range 0-82). Seventeen women (57.5%) had a negative CAT, whereas 23 women (42.5%) had a positive CAT.
All women took care of appropriate contraception (condoms or no intercourse) in the month that laparoscopy was performed. None of the patients used oral contraceptives. Laparoscopy reports were scored independently by two investigators who were unaware of the CAT results, and were categorized according to the presence of adhesions and tubal patency. Of all 40 women, 31 women (77.5%) had no tubal pathology and 9 women (22.5%) had tubal pathology. Of the 31 women without tubal pathology, 6 had a history of abdominal surgery (four uneventful appendectomies, one Caesarean section and one uneventful appendectomy and ileocoecal resection because of Crohn's disease) and 2 had a history of proven and treated *C. trachomatis* cervicitis. Of the 9 women with tubal pathology, 6 had previous abdominal surgery (one ectopic pregnancy following an episode of suspected and treated PID, one extensive surgery for endometriosis, one unilateral cystectomy, one uneventful appendectomy and repeated coagulation of endometriosis and two uneventful appendectomies).

Immunohistochemical staining

For the purpose of immunohistochemical staining, sections of 5 μm thickness were cut from the paraffin-embedded blocks.

Monoclonal anti-MOMP and anti-HSP antibodies
Paraffin sections were deparaffinized in xylene for 2 * 5 minutes and rehydrated in alcohol series. Endogenous peroxidase was blocked by incubation with 0.25% hydrogen peroxide in methanol for 25 minutes. Sections were washes three times in phosphate-buffered saline (PBS). Antigen retrieval was performed by microwave irradiation in sodium citrate pH 6.0 for 20 minutes, followed by cooling for 20 minutes. Sections were washed three times in PBS and preincubated with 2% bovine serum albumin (BSA) in PBS for 10 minutes. Sections were incubated overnight at 4°C with the primary monoclonal anti-MOMP antibody (1:1500; Fitzgerald Industries International, Inc., Concord, USA) or were incubated for two hours at 4°C with the primary monoclonal anti-HSP antibody (1:750; Affinity BioReagents, Golden, USA) respectively. After washing once in 0.1% Tween20 in PBS and twice in PBS (in case of the anti-HSP antibody: three times in PBS), sections were exposed to ChemMate DAKO EnVision (DakoCytomation, Glostrup, Denmark) for 30 minutes. After washing once in 0.1% Tween20 in PBS and twice in PBS (in case of the anti-HSP antibody: three times in PBS), antibody binding was visualized by incubation with 3,3'-diaminobenzidine (DAB) for 7 minutes. Sections were washed in Milli-Q, counterstained in haematoxylin, washed in tap water, dehydrated and mounted in Entellan for light microscopic evaluation.

Polyclonal anti-MOMP antibodies
Paraffin sections were deparaffinized in xylene for 2 * 5 minutes and rehydrated in alcohol series. Endogenous peroxidase was blocked by incubation with 0.25% hydrogen peroxide in methanol for 25 minutes. Sections were washes three times in PBS. Antigen retrieval was performed by microwave irradiation in sodium citrate pH 6.0 for 20 minutes, followed by cooling for 20 minutes. Sections were washed three times in PBS and preincubated with 2% BSA in PBS for 10 minutes. Sections were incubated overnight at 4°C with the primary polyclonal antibodies (both 1:3000; Fitzgerald Industries International, Inc., Concord, USA and US Biological, Swampscott, USA). After washing once in 0.1% Tween20 in PBS and twice in PBS, sections were incubated with the secondary polyclonal biotinylated rabbit anti-goat antibody (1:100; DakoCytomation, Glostrup, Denmark) for 30 minutes. After washing once in 0.1% Tween20 in PBS and twice in PBS, sections were exposed to ChemMate DAKO EnVision (DakoCytomation, Glostrup, Denmark) for 30 minutes. After washing once in 0.1% Tween20 in PBS and twice in PBS, antibody binding was visualized by incubation with DAB for 7 minutes. Sections were washed in Milli-Q, counterstained in haematoxylin, washed in tap water, dehydrated and mounted in Entellan for light microscopic evaluation.

PCR
Genomic DNA was extracted from the paraffin-embedded endometrial tissue using the High Pure PCR Template Preparation Kit according to the manufacturers' instructions (Roche Molecular Biochemicals, Grenzach-Wyhlen, Germany). *C. trachomatis* detection was performed using an in-house real time PCR-based TaqMan-assay, as described elsewhere (Catsburg *et al.*, 2006).

Positive and negative controls for immunohistochemical staining and PCR
Human epithelial cells (HEp2, ATCC CCL-23) were cultured in culture medium [Eagle's Minimal Essential Medium (Cambrex BioScience, Verviers, Belgium) supplemented with 10% fetal calf serum, non-essential aminoacids and 200 mM L-Glutamin] at 37˚C with 5% CO_2 and saturated humidity. The HEp2 positive controls were inoculated with *C. trachomatis* (human serovar L2) at a multiplicity of infection of 1 and diluted in infection medium [Eagle's Minimal Essential Medium (Cambrex BioScience, Verviers, Belgium) supplemented with 2% fetal calf serum, non-essential aminoacids, 200 mM L-Glutamin and 0.5 ug/mL cycloheximide]. Cells were incubated for one hour at room temperature and 1900 rounds per minute, and subsequently cultivated at 37˚C. The HEp2 negative controls were inoculated with sucrose-phosphate-glucose solution in infection medium and processed in the same manner as the HEp2 positive controls. Finally, the cells were harvested and embedded in paraffin.

Scoring methods

Two investigators (F.R.M.S. and S.A.M.), who were unaware of the CAT results and laparoscopy reports, independently scored all 160 patient sections (40 patients * 4 antibodies) and the positive and negative HEp2 controls. A semi-quantitative scoring method was developed and applied in multiple high power fields (* 400). The following consensus-based definitions were used: no cells stained = negative (score 0); less than five cells stained = equivocal (score 1); five or more cells to a maximum of 50% of all cells stained = positive (score 2); more than 50% of all cells stained = highly positive (score 3). Sections in which it was impossible to quantify the staining score (e.g. extensive background staining, too little tissue or folded sections) were excluded from the analyses.

Statistical analysis

Kappa was calculated to measure the agreement in staining score between both observers for each of the four antibodies in sections in which both investigators were able to quantify the staining score. For this purpose, the semi-quantitative scoring method had to be dichotomized. For all four antibodies, we have calculated

kappa-values twice: once with equivocal sections categorized as positive and once with equivocal sections categorized as negative. Kappa-values between 0 and 0.19 indicate poor agreement, values between 0.20 and 0.39 indicate fair agreement, values between 0.40 and 0.59 indicate moderate agreement, values between 0.60 and 0.79 indicate substantial agreement and values between 0.80 and 1.00 indicate (almost) perfect agreement between both observers.
For a comparison between immunohistochemical staining results and clinical parameters (CAT and tubal status), a mean staining score was calculated for each section for the four antibodies separately. Furthermore, a total mean staining score was calculated for each section, i.e.the sum of the staining scores for all four antibodies separately and for both observers divided by the number of sections in which the staining could be quantified. The Mann-Whitney *U*-test was used to compare the mean staining score in CAT-positive and CAT-negative women. The Kruskal-Wallis test was used to compare the mean staining score in CAT-positive and CAT-negative women with and without tubal pathology. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

Immunohistochemical staining

The positive controls (*C. trachomatis* infected HEp2 cells) were all highly positive with all four antibodies used in this study (Figure I), whereas the negative controls (mock-infected HEp2 cells) were all negative (Figure II).
The percentage of endometrial sections in which the staining could be quantified was 79%, 80%, 78% and 34% for the monoclonal anti-MOMP antibody (Fitzgerald Industries International), both polyclonal anti-MOMP antibodies (Fitzgerald Industries International and US Biological) and the monoclonal anti-HSP antibody (Affinity BioReagents) respectively.
Kappa was calculated to document inter-observer variability. First, equivocal sections were categorized as positive. Kappa was 0.93, 1.00 and 0.92 for the monoclonal anti-MOMP antibody and both polyclonal anti-MOMP antibodies respectively, indicating (almost) perfect agreement between both observers. For the monoclonal anti-HSP antibody, kappa was 0.35, indicating moderate agreement between both observers. If equivocal sections were categorized as negative, kappa was 0.90, 0.89, 0.70 and 0.74 for all four antibodies respectively. In the present study, the intra-observer variability was not determined.

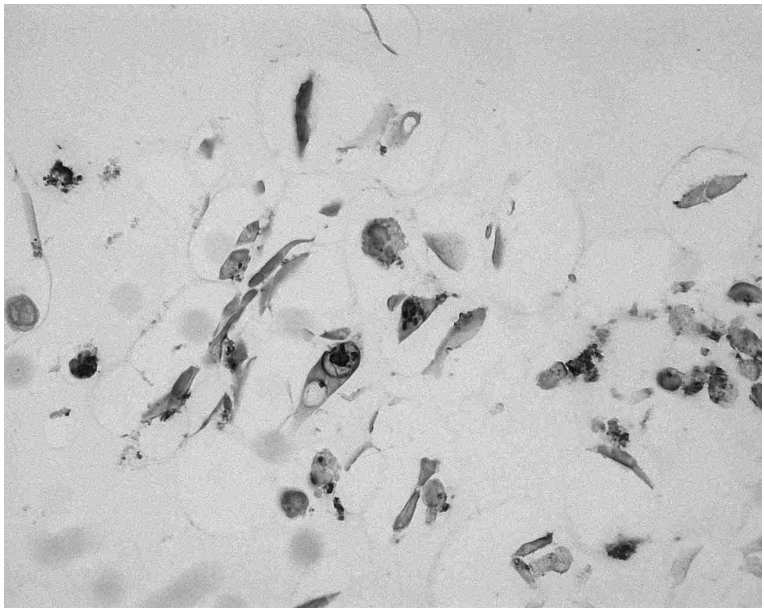

Figure I. *C. trachomatis*-infected HEp2 cells stained with anti-MOMP antibody (1:1500; Fitzgerald Ind. Int.) (* 400).

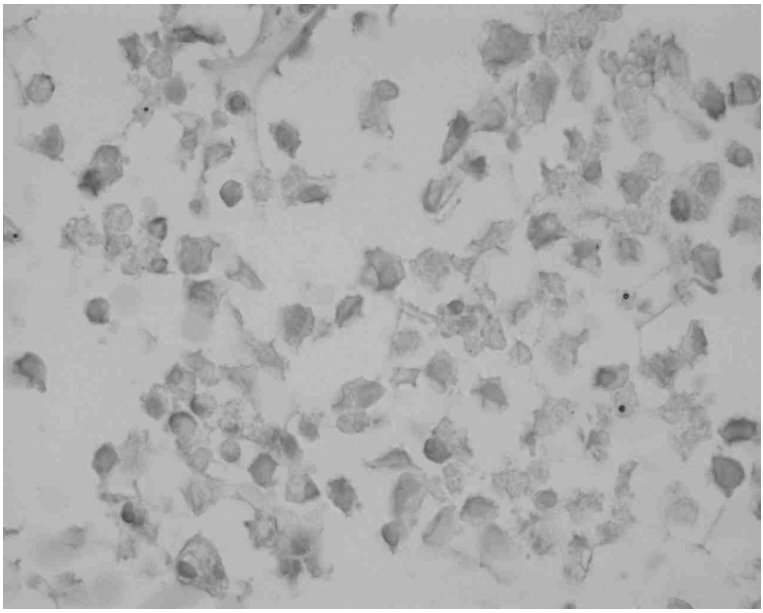

Figure II. Mock-infected HEp2 cells stained with anti-MOMP antibody (1:1500; Fitzgerald Ind. Int.) (* 400).

Table IV and V reflect the relation between clinical parameters (CAT and the presence of tubal pathology) and the mean staining score. When comparing the mean staining scores in CAT-negative and CAT-positive women, respectively, no significant differences were found (Table IV). Also, when taking the tubal status into account, no significant differences were found in staining scores between the subgroups mentioned (Table V).

Table IV. Relation between CAT and mean staining score.

CAT[a]	Mean staining score[b]				
	Monoclonal anti-MOMP antibody (Fitzgerald Ind. Int.)	Monoclonal anti-HSP antibody (Affinity Bio-Reagents)	Polyclonal anti-MOMP antibody (Fitzgerald Ind. Int.)	Polyclonal anti-MOMP antibody (US Biological)	All four antibodies combined
Negative	0.9	1.0	0.8	0.8	0.8
Positive	0.7	1.2	0.3	0.7	0.6

[a] *C. trachomatis* IgG antibody test; [b] Mean staining score: no cells stained = negative (score 0); less than five cells stained = equivocal (score 1); five or more cells to a maximum of 50% of all cells stained = positive (score 2); more than 50% of all cells stained = highly positive (score 3).

Table V. Relation between CAT, tubal status and mean staining score.

CAT[a]	TP[b]	n	Mean staining score for all four antibodies combined[c]
Negative	No	17[d]	0.7
Negative	Yes	5	0.9
Positive	No	12[e]	0.6
Positive	Yes	4	0.5

[a] *C. trachomatis* IgG antibody test; [b] Tubal pathology; [c] Mean staining score: no cells stained = negative (score 0); less than five cells stained = equivocal (score 1); five or more cells to a maximum of 50% of all cells stained = positive (score 2); more than 50% of all cells stained = highly positive (score 3); [d] Exclusion of 1 CAT-negative patient: staining could not be quantified in all sections; [e] Exclusion of 1 CAT-positive patient: staining could not be quantified in all sections.

PCR

The positive controls (*C. trachomatis* infected HEp2 cells) were positive in the PCR analysis, whereas the negative controls (mock-infected HEp2 cells) were negative. From 13 women (not including the three patients with a history of suspected PID or *C. trachomatis* cervicitis), sufficient endometrial tissue was available for PCR analysis. Of these 13 women, five women (38%) had a negative CAT and no tubal pathology, two women (15%) had a negative CAT and tubal pathology, four women (31%) had a positive CAT and no tubal pathology and two women (15%) had a positive CAT and tubal pathology. In these 13 endometrial samples, the mean staining score was 1.0, 0.8, 0.8 and 1.5 when using the monoclonal anti-MOMP antibody (Fitzgerald

Industries International), both polyclonal anti-MOMP antibodies (Fitzgerald Industries International and US Biological) and the monoclonal anti-HSP antibody (Affinity BioReagents) respectively. Using PCR, all 13 endometrial samples tested were *C. trachomatis* negative, while the control beta globin PCR checking for the presence of human DNA was all positive in all samples.

DISCUSSION

It is known that *C. trachomatis* is able to persist in the fallopian tubes of women with tubal pathology (Patton *et al.*, 1994, Lan *et al.*, 1995, Haeusler *et al.*, 1997, Gérard *et al.*, 1998, Noguchi *et al.*, 2002). It is hypothesized that the endometrium may also harbour persistent *C. trachomatis* micro-organisms, which may negatively affect implantation rates. In this pilot study, we have evaluated the detection rate of *C. trachomatis* in paraffin-embedded endometrial samples of subfertile women, using immunohistochemical staining and PCR.
Of all 40 women participating in the present study, 42.5% had serological evidence of a previous *C. trachomatis* infection (as reflected by the presence of *C. trachomatis* IgG antibodies in serum) and 22.5% had tubal pathology at laparoscopy.
Immunohistochemical staining resulted in moderately to well-consistent findings, as reflected by satisfactory kappa-values (0.35—1.00). However, no significant relation was found between the staining results and clinical parameters (CAT and tubal status). Possible explanations for our findings may be that *C. trachomatis* was not present in the endometrium, it was missed in the sample or in the slices, or smaller pieces of the micro-organism were present, which immunohistochemical staining methods fail to detect. Furthermore, the staining that was observed in the endometrial samples may have been aspecific. In approximately 1 out of 5 sections (for the monoclonal anti-HSP antibody even in 2 out of 3 sections), we were not able to quantify the staining appropriately, indicating that this test method is of little use.
Because of the absence of a correlation between staining results and clinical parameters, we have decided to perform PCR, which is known to be a more accurate test method as compared to immunohistochemical staining, on part of the samples. The decision to perform immunohistochemical staining as a first test was made at the start of our pilot study, because we did not have sufficient tissue available for PCR on all samples. PCR (which is considered the most accurate test method) showed negative results in all 13 samples tested, while the control beta globin PCR checking for the presence of human DNA was positive in all samples. Different factors may explain our negative PCR results. It is possible that *C. trachomatis* was present in the endometrium, but it was missed when collecting the biopsy samples or cutting the slices. In literature, varying prevalence results (3-50%; Table III) are described, and our population may be too small to include *C. trachomatis*-positive

cases. Unfortunately, no sufficient endometrial tissue was available for PCR testing in the remaining 27 women.

Furthermore, all women received prophylactic antibiotics (a single oral dose of 1000 mg azithromycin on the evening preceding laparoscopy) to protect against iatrogenic spread or reactivation of unnoticed *C. trachomatis* genital tract infections during instrumentation (Land *et al.*, 2002). Azithromycin is known for its ability to rapidly reach very high tissue concentrations (within a few hours) (Lode, 1991, Peters *et al.*, 1992, Worm and Østerlind, 1995, McCarty, 1996) and to inhibit protein synthesis in *C. trachomatis*-infected cells (Engel, 1992). When administered orally preceding gynaecological surgery, highest tissue levels of azithromycin have been demonstrated after 24 hours, and even after up to 96 hours azithromycin has still been detectable in gynaecological tissues (Krohn, 1991). After administration of a single oral dose of 1000 mg azithromycin to patients with a *C. trachomatis* genital tract infection, frequent PCR testing has revealed a clearance rate of 32% after one day and 60% after one to three days (Bianchi *et al.*, 1998, Gaydos *et al.*, 1998), although *C. trachomatis* DNA may remain detectable for three to four weeks after antibiotic treatment (Morré *et al.*, 1998). This indicates that the negative PCR results in our study may be explained by clearance following prophylactic treatment with azithromycin.

Implantation rates could not be studied, because only part of our patients eventually underwent IVF, which is the only treatment which allows for calculation of implantation rates. To our knowledge, of all 40 women studied, 29 (73%) eventually conceived either spontaneously or following fertility treatment. The remaining 11 patients did not conceive despite fertility treatment (n = 2), underwent fertility treatment in another hospital (n = 2), discontinued fertility treatment (n = 2 because of poor ovarian response, n = 1 because of fertilization failure at IVF and n = 1 for personal reasons) or were lost to follow-up (n = 3). Remarkably, for all four antibodies studied the mean staining score was lower in women who eventually conceived as compared to those who did not conceive, and this difference was statistically significant for the monoclonal anti-MOMP antibody (Fitzgerald Industries International) (data not shown). The pregnancy rate in the subgroup of *C. trachomatis* PCR-negative women was 77% (10/13). The hypothesis that endometrial involvement in *C. trachomatis* infections may influence implantation therefore still deserves further consideration. Future research may focus on PCR-testing of endometrial samples in a larger prospective cohort study, in which CAT, tubal status and implantation rates at IVF will be available.

In summary, the aim of the study was to evaluate the detection rate of C. *trachomatis* in endometrial samples of subfertile women with and without tubal factor subfertility using two different test methods (immunohistochemical staining and PCR). In our hands and with the antibodies used, no correlation was shown between immunohistochemical staining results and clinical parameters in our cohort of 40 pa-

tients. PCR results were negative in all 13 samples tested, but our study population was very small. Our hypothesis may be retested in a larger cohort using PCR, clinical parameters (CAT and tubal status) and clinical outcome (implantation rates).

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Chapter 9
General discussion

The topic of this thesis is persistent *Chlamydia* (*C.*) *trachomatis* infections and the ensuing tubal factor subfertility. How do we diagnose persistent *C. trachomatis* infections and tubal pathology, respectively, in subfertile women? And why does *C. trachomatis* persist? And do persistent *C. trachomatis* infections also influence endometrial receptivity?
In Chapters 2—4, different screening tests that can be used to estimate the risk of tubal factor subfertility have been evaluated. Chapters 5—7 have addressed the immunogenetic contribution to persistence of *C. trachomatis* infections. Chapter 8 has described different methods to detect persistent *C. trachomatis* infections in endometrial tissue of subfertile women, serving as a starting point for future studies on the association between *C. trachomatis* endometritis and subfertility.
The current chapter will highlight and discuss the present insights on persistent *C. trachomatis* infections and tubal factor subfertility, including the findings as described in this thesis and recommendations for further studies.

PREVALENCE, CLINICAL MANIFESTATIONS AND COMPLICATIONS

C. trachomatis is the most prevalent sexually transmitted disease in industrialized countries. Among the risk factors for acquiring *C. trachomatis* genital tract infections are a high level of urbanization, young age, low education, multiple lifetime sexual partners, a recent new sexual partner and recent unprotected sexual intercourse (Götz *et al.*, 2005). Figure I represents the clinical course and outcome of *C. trachomatis* infections.

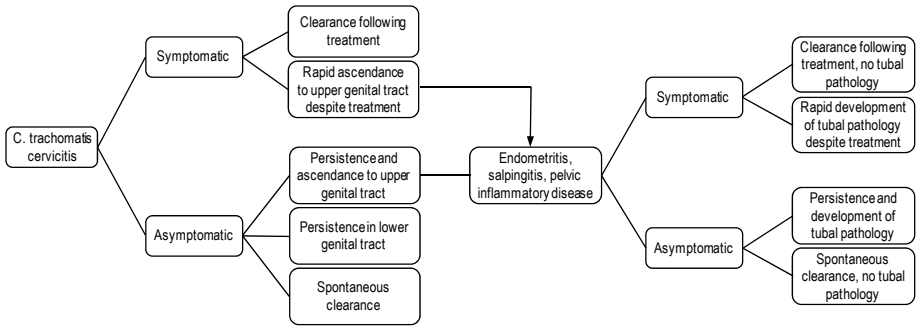

Figure I. Clinical course and outcome of *C. trachomatis* infections.

In the Netherlands, 35 000 women are infected by *C. trachomatis* annually (Health Council of The Netherlands, 2005). The majority of these women have lower genital tract infections, which often remain asymptomatic. Spontaneous clearance rates of ~ 50% in the first year and of up to 94% in five years have been reported (Morré *et*

al., 2002, Molano *et al.*, 2005). *C. trachomatis* lower genital tract infections seem to have no influence on the patient's fertility (Andersen *et al.*, 2005). However, in the Netherlands each year an estimated 5 000 to 10 000 women develop upper genital tract infections, which are also asymptomatic in most women. The risk of subfertility following *C. trachomatis* upper genital tract infections varies between 0.6 and 40%, depending on the number and severity of infections (Weström *et al.*, 1992). *C. trachomatis*-associated tubal factor subfertility affects 1 000 to 2 000 women each year in the Netherlands (Health Council of The Netherlands, 2005), which is in concordance with the findings of Land and co-workers (2010), who have estimated that the risk of tubal factor subfertility following a *C. trachomatis* lower genital tract infection is up to 4.6%.

The actual risk of subfertility due to *C. trachomatis* infections may even be higher, because women with persistent subclinical *C. trachomatis* endometritis — but patent tubes — may have impaired embryo implantation capacities and therefore lower pregnancy rates (Witkin *et al.*, 1994, Witkin *et al.*, 1995, Kamiyama *et al.*, 2004, Romero *et al.*, 2004, Pacchiarotti *et al.*, 2009). Recent *in vitro* studies have shown that *C. trachomatis* infections are able to influence the metabolism of the trophoblast, which may not only affect implantation capacities, but may also be associated with adverse pregnancy outcomes such as preterm labor or pre-eclampsia (Azenabor *et al.*, 2007, De la Torre *et al.*, 2009).

Endometrial sampling is not performed routinely in the fertility work-up any longer, and therefore women with *C. trachomatis* endometritis may now be wrongly categorized as having an unexplained subfertility. The pilot study for the detection of *C. trachomatis* in endometrial tissue of subfertile women, as described in Chapter 8, did not yield positive results, but can be considered as a first step towards a larger prospective study collecting data on patient characteristics, serological screening, endometrial sampling, tubal status and clinical outcome in order to retest the hypothesis that *C. trachomatis* endometritis may contribute to subfertility. Preferably, this is to be studied in a cohort of women undergoing IVF for both tubal and non-tubal factor subfertility, respectively, in order to study pregnancy rates and implantation rates most accurately. If our hypothesis could be confirmed in further studies, it should also be tested whether antibiotic treatment would improve fertility in women with *C. trachomatis* endometritis. Women with subacute endometritis significantly benefit from antibiotic treatment with respect to clinical symptoms (e.g. abnormal bleeding, mucopurulent cervicitis and uterine tenderness) and histological findings (Eckert *et al.*, 2004), but it is unclear whether fertility would improve by antibiotic treatment.

SCREENING FOR *C. TRACHOMATIS*-ASSOCIATED TUBAL FACTOR SUBFERTILITY

As described in Chapters 2—4, *C. trachomatis* IgG antibody testing (CAT) remains the best method to accurately discern subfertile women with a high versus low risk of tubal pathology. In our population, with a prevalence of distal tubal pathology of 18.8%, the positive predictive value of CAT was 62%, whereas the negative predictive value was 90%. Our results on the value of CAT are in agreement with findings in literature (as summarized by Den Hartog *et al.*, 2006). Following a negative CAT, tubal testing by hysterosalpingography (HSG) may be performed because of its high specificity and fertility-enhancing effect (Chapter 4), although HSG does not help in discerning women with a high versus low risk of tubal pathology, respectively. In CAT-negative women, the probability of tubal pathology was 14%, and the probability ranged between 13 and 17% after performing HSG .

We have been the first, in 2005, to report that measuring high sensitivity C-reactive protein (hs-CRP) levels significantly increases the predictive value of CAT for tubal pathology (Chapter 2), with an increase in odds ratio from 13.9 to 39.7, although larger studies are needed to confirm that hs-CRP testing deserves a role in the fertility work-up. Two studies, published since then, have now also reported a higher risk of late sequelae in women with previous *C. trachomatis* infections and elevated hs-CRP levels. Agrawal and co-workers (2007) have studied local and serological immune responses in 320 women with a previous *C. trachomatis* infection in relation to fertility disorders. Elevated hs-CRP levels, which are considered to reflect chronic inflammation, were significantly more prevalent in women with fertility disorders (46%) as compared to those without fertility disorders (23%). Combining hs-CRP (as a serological marker of chronic inflammation) and interferon gamma (IFN-γ; as a local marker of infection) resulted in a significantly higher predictive value for fertility disorders as compared to IFN-γ alone (OR 37.9 versus 15.4) (Agrawal *et al.*, 2007). Karinen and co-workers (2005) have studied the association between persistent *C. trachomatis* infections, as tested in the first trimester of pregnancy, and the risk of preterm delivery in singleton pregnancies. A comparison was made between 402 term deliveries and 104 preterm deliveries (mean gestational age 34.4 weeks, range 26-36 weeks). The simultaneous presence of *C. trachomatis* IgG antibodies and elevated hs-CRP levels increased the risk of preterm delivery more than fourfold in comparison to women with no or only one marker present (Karinen *et al.*, 2005).

Several other markers of persistence (*C. trachomatis* IgA antibodies, heat shock protein 60 IgG antibodies and lipopolysaccharide IgG antibodies) have also been found to be significantly more prevalent in women with tubal pathology, although their performance was not as good as the performance of CAT (Chapters 2 and 3). Furthermore, no significant additive role of other *Chlamydiaceae* species in the

development of tubal pathology could be established (Chapter 3), although it was hypothesized that other species could contribute in the pathogenesis of tubal damage by eliciting a genus-specific auto-immune inflammatory response. This hypothesis has recently been supported by Dean and co-workers (2008), who have demonstrated that one-third of the patients with trachoma has a mixed infection with multiple *Chlamydiaceae* species, and have suggested that these other species may also induce an inflammatory response leading to ocular scarring. Therefore, the discussion on the role of various *Chlamydiaceae* species on the course and outcome of *C. trachomatis* infections is still open.

INTER-PATIENT VARIABILITY IN COURSE AND OUTCOME OF *C. TRACHOMATIS* INFECTIONS

Differences in course and outcome of *C. trachomatis* infections are determined by virulence factors of the pathogen, environmental factors and host immune factors. Carrying genetic variations in the innate immune system seems to be the most important determinant of the inter-patient variability in course and outcome of *C. trachomatis* infections (Chapter 1, Morré *et al.*, 2009). In a recent study, Bailey and co-workers (2009) have also confirmed that host genetic factors contribute largely (~ 40%) to the course of *C. trachomatis* infections.

Cells of the innate immune system possess pattern recognition receptors (PRRs), which are able to recognize foreign micro-organisms, and subsequently destroy them (by ingestion of the micro-organism or by cytolysis). Carrying genetic variations in genes encoding PRRs [e.g. toll-like receptors (TLRs) and caspase recruitment domains (CARDs)/nucleotide oligomerization domains (NODs)] may negatively affect the immune response following *C. trachomatis* infections, resulting in a higher risk of developing tubal pathology (as summarized in Chapter 1). Chapter 7 shows a trend towards a higher risk of tubal factor subfertility in CAT-positive carriers of single nucleotide polymorphisms (SNPs) in genes encoding TLR9, TLR4 and CARD15/NOD2, especially in those patients carrying at least two SNPs (73% risk of tubal pathology, in comparison to 33% in women carrying less than two SNPs). Cluster of differentiation (CD) 14, co-receptor of TLR4, does not play a role in susceptibility to or outcome of *C. trachomatis* infections (Chapters 6 and 7). In Chapter 5, a knockout mouse model was designed to study the role of TLR4 in *C. trachomatis* infection and re-infection. In *TLR4* knockout mice, clearance of *C. trachomatis* occurred slower as compared to *TLR4* wildtype mice. This difference was more solid in case of repeated *C. trachomatis* infections. Carrying a *TLR4* SNP did not significantly influence the susceptibility to or outcome of *C. trachomatis* infections (Chapter 5).

It has also been known that genetic variations in genes encoding cytokines, which are produced by cells of the innate immune system following recognition of a

pathogen, can be associated with alterations in the course and outcome of infectious diseases. A recent study in 114 women with *C. trachomatis*-associated subfertility and 176 controls has shown that carrying a genetic variation in the gene encoding tumour necrosis factor alpha (*TNF-α* -308 G>A) significantly increases the risk of severe tubal damage (odds ratio (OR) of 4.0 for *TNF-α* 308 A allele) (Öhman *et al.*, 2009). Other studies have also found a significant association between *TNF-α* -308 G>A SNP and the risk of trachoma (as reviewed by Morré *et al.*, 2009). Despite these positive findings, no significant difference in *TNF-α* 308 genotype could be found between 35 CAT-positive women with tubal factor subfertility (4 carriers of the A-allele) and 35 CAT-negative women with tubal factor subfertility (6 carriers of the A-allele) in a previous study (Cohen *et al.*, 2003). The latter study, however, might be too small to find significant differences, and did not include a control group of women without tubal pathology or without subfertility.

In contrast, SNPs in other genes seem to act in a protective way, i.e. they protect the host from tubal damage related to hyperinflammation. An example of this is a 32 basepair deletion in the gene encoding chemokine (C-C motif) receptor (CCR) 5, which is a cytokine receptor on cells of the immune system. Among CAT-positive women, the *CCR5d32* deletion was present in 31% of women without tubal pathology and in only 7% of women with tubal pathology (OR 5.8, $P < 0.05$) (Barr *et al.*, 2005). Carrying the interleukin (IL) 10 -1082 A>G SNP has also been shown to be protective against tubal pathology (OR for tubal pathology 7.3 for the wildtype IL-10-1082AA genotype) (Öhman *et al.*, 2009) and against trachoma (as summarized by Morré *et al.*, 2009).

Further immunogenetic studies will help to elucidate the pathophysiology of *C. trachomatis* infections and to explain the inter-patient variability in course and outcome of *C. trachomatis* infections. The ultimate goal is to predict, without using invasive techniques, which subfertile women do or do not have tubal pathology following *C. trachomatis* infection. The European funded EpiGenChlamydia Consortium (see www.EpiGenChlamydia.EU), composed of 20 Universities and Institutes in Europe, Africa and the US, is developing Biobanks and data-warehouses to perform large scale host immunogenetic studies for obtaining genetic traits which - combined with serological markers - can be used for patient profiling. The various stakeholders in Public Health play a key role in translating the implications of genomics derived from molecular epidemiology and host-pathogen genomics. This knowledge will enable not only clinical interventions, but also health promotion messages and disease prevention programmes to be targeted at susceptible individuals as well as subgroups of the population based on their genomic profile (personalized healthcare) (Brand, 2009). The field involved in this translation is called Public Health Genomics, which has as major task *"the responsible and effective translation of genome-based knowledge and technologies into public policy and*

health services for the benefit of population health" (Bellagio statement, 2005: see www.graphint.org for details).

PRIMARY, SECONDARY AND TERTIARY PREVENTION OF *C. TRACHOMATIS* INFECTIONS

Primary prevention of *C. trachomatis* infections mainly consists of public campaigns focussing on health education to prevent exposure to sexually transmitted infections and to increase awareness of the early symptoms of *C. trachomatis* infections. On an individual level, this education — as well as opportunistic screening — can also be supplied by general practitioners, e.g. when adolescents consult them for advise on contraceptive methods. Municipal Health Services are low-barrier health care suppliers, mainly occupied with the implementation of large population-based screening programmes and contact tracing, thereby supporting primary and secondary prevention measures. As long as an effective vaccine against *C. trachomatis* is not available, primary prevention is limited to the methods as described (Brunham and Rey-Ladino, 2005).

The goal of secondary prevention strategies is to diagnose *C. trachomatis* infections in an early stage, in order to immediately start treatment and prevent late complications. General practitioners play an important role in secondary prevention since they are easily accessible. The majority of women with symptoms suspected for a sexually transmitted disease turn to their general practitioner, and almost half of the total number of *C. trachomatis* tests which are processed by Dutch laboratories come from general practitioners (Health Council of The Netherlands, 2004). Nowadays, self-collected vaginal or urinary samples in combination with internet-based questionnaires have also shown to be accurate and acceptable methods to screen for *C. trachomatis* (Götz *et al.*, 2005, Gaydos *et al.*, 2006). Ninety percent of the participants of internet-based screening programmes (via websites such as www.iwantthekit.org) prefer self-collection of the samples and rate self-collection as easy or very easy (Gaydos *et al.*, 2006). Low-barrier internet-based screening programmes thereby facilitate the detection and treatment of *C. trachomatis* infections, although the precise impact of screening programmes on the prevalence of tubal factor subfertility remains to be studied more precisely (Land *et al.*, 2010).

Tertiary prevention strategies aim to minimize the risk of complications following *C. trachomatis* infections. The asymptomatic course of most *C. trachomatis* infections results in a disappointing role for tertiary prevention, because usually substantial tubal damage has already occurred by the time symptoms develop, or the patient has already presented with subfertility many years later (Paavonen and Lehtinen, 2006).

SUMMARY AND FUTURE PERSPECTIVES

Subfertile couples seeking medical treatment are usually unaware of a previous *C. trachomatis* genital tract infection. Direct and non-invasive testing for the presence of *C. trachomatis* or signs of chronic inflammation is possible in only part of the genital tract. However, most subfertile women do not fulfill the risk profile of current infection, and indeed the prevalence of *C. trachomatis* lower genital tract infections in subfertile women has been shown to be low (Chapter 8). Also, since *C. trachomatis* cervicitis does not seem to influence fertility, cervical sampling — although easy to perform — does not contribute to the risk assessment of tubal pathology. The endometrium is also accessible for investigation, but the prevalence and precise role of *C. trachomatis* endometritis in subfertility (diminished implantation capacity?) is still unclear and needs further evaluation (Chapter 8). Furthermore, it is unknown whether antibiotic treatment improves fertility in women with *C. trachomatis* endometritis.
The reference standard for tubal testing is laparoscopy, but should preferably be reserved for women with a high risk of tubal pathology. Conjoint analysis studies have to determine how gynaecologists value different features of a patient (e.g. age, duration of subfertility, previous pregnancy, suspicion of intra-abdominal pathology including endometriosis and results of screening tests in the fertility work-up) in the decision whether laparoscopy should be performed. Tubal patency can be tested by flushing the tubes with methylene blue dye during laparoscopy. However, this does not reflect the intraluminal status (damaged epithelial lining? impaired ciliary function?). Obtaining biopsies of the tubes should only be conducted for research purposes in women in whom tubectomy is necessary, whereas it does not deserve a place in the regular fertility investigations.
Screening strategies for *C. trachomatis*-associated tubal factor subfertility should at least comprise chlamydia antibody testing (CAT), and — in low-risk women only — HSG may be performed (Chapters 2—4). Currently, individual patient data meta-analyses are being performed to re-evaluate the precise value of both CAT and HSG (Broeze *et al.* (a), *in preparation*, Broeze *et al.* (b), *in preparation*). In addition to CAT, serological markers of persistence, such as hs-CRP, may help to estimate the risk of tubal factor subfertility more accurately (Chapter 2). Laparoscopy can then be reserved for subfertile patients with the highest risk of tubal pathology, as assessed by serological screening tests.

Immunogenetic studies strongly support the hypothesis that the immune system plays a key role in the course and outcome of *C. trachomatis* infections (Chapters 5—7) with a genetic predisposition of 40%. Elucidating which genes are responsible for this 40% genetic predisposition in course and outcome of *C. trachomatis* infections is a major challenge, which will provide potential clinical relevant genetic SNP

traits for patient management. Large scale initiatives are being employed by the European funded EpiGenChlamydia Consortium (www.EpiGenChlamydia.EU) which was recently linked to the field of Public Health Genomics (www.graphint.org, www.ecphg.eu and www.phgen.eu) to further explore the potential opportunities.

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Summary

Chlamydia (*C.*) *trachomatis* is the most prevalent sexually transmitted disease in industrialized countries, affecting 35 000 women in the Netherlands each year. Because most women do not experience symptoms and, therefore, do not receive antibiotic treatment, *C. trachomatis* infections may ascend to the upper genital tract and may persist for many years, increasing the risk of late complications. Up to 4.6% of women with *C. trachomatis* lower genital tract infections will eventually develop tubal factor subfertility. The reference standard for diagnosing tubal pathology in subfertile women is laparoscopy with tubal testing. In order to prevent exposure to invasive tubal testing of all subfertile women, screening methods — comprising serological testing and hysterosalpingography (HSG) — are used to discern subfertile women with a high and low risk of tubal pathology, respectively.

An introduction to the subject, as well as the outline of this thesis, was provided in **Chapter 1**. The first aim of this thesis was to optimize the screening strategy for diagnosing persistent *C. trachomatis* infections and the ensuing tubal pathology in subfertile women. The second aim was to study the immunogenetic contribution to persistence of *C. trachomatis* infections. The third aim was to assess different test methods to detect persistent *C. trachomatis* infections in endometrial tissue of subfertile women.

Persistent *C. trachomatis* infections are assumed to be important risk factors for tubal pathology. In **Chapters 2** and **3**, several serological markers of persistence were studied in a cohort of 313 subfertile women, who all underwent laparoscopy with tubal testing to assess the tubal status. Besides the currently used screening test [*C. trachomatis* immunoglobulin (Ig) G antibody testing; CAT], IgG antibodies to chlamydia heat shock protein 60 (cHSP60) and to chlamydia lipopolysaccharide (LPS), IgA antibodies to *C. trachomatis* and high-sensitivity CRP (hs-CRP) were measured. The prevalence of tubal pathology — as defined as extensive peri-adnexal adhesions and/or distal occlusion of at least one tube at laparoscopy — was 18.8%. All serological markers of persistence were significantly more prevalent in women with tubal pathology as compared to those without tubal pathology. CAT remained the best single test [odds ratio (OR) 13.9]. Only the combination of CAT (as a marker of a previous *C. trachomatis* infection) and hs-CRP (as a marker of persistence) resulted in a significantly higher OR (39.7), and therefore this test combination is a candidate screening method in the fertility work-up.

In the same study cohort, the role of other *Chlamydiaceae* species in the development of tubal pathology was evaluated (**Chapter 3**). It was hypothesized that *C. pneumoniae* and *C. psittaci* contribute in the pathogenesis of tubal damage by eliciting a genus-specific auto-immune inflammatory response, but our findings could not support this hypothesis.
In **Chapter 4**, three different screening strategies (comprising CAT, hs-CRP and HSG) were explored in a population of 642 subfertile women who all underwent tubal testing (HSG and/or laparoscopy). The goal of this observational study was to develop a screening strategy which is able to accurately estimate the risk of tubal pathology in a non-invasive manner. Based on our findings, we proposed CAT as the first screening test for tubal pathology in subfertile women (risk of tubal pathology 14% in CAT-negative women and 53% in CAT-positive women, respectively). Hs-CRP seemed promising, and the value of HSG was shown to be limited. We suggested to omit laparoscopy in CAT-negative patients and in patients with normal or inconclusive HSGs, whereas in CAT-positive women and in women with abnormal HSGs, laparoscopy remains justified.

Variations in immunologically important host genes are assumed to result in an aberrant immune response against *C. trachomatis* infections, thereby increasing the risk of persistence and tubal pathology. **Chapter 5** described a *toll-like receptor* (*TLR*) *4* knockout mouse model and human candidate gene approach. In *TLR4* knockout mice, clearance of *C. trachomatis* infection and re-infection occurred slower as compared to *TLR4* wildtype mice, indicating a role of TLR4 in the immune response to *C. trachomatis* infections. In 614 women visiting a Sexually Transmitted Diseases outpatient clinic and in 259 subfertile women, carrying the *TLR4* +896 A>G single nucleotide polymorphism (SNP) did not play a significant role in susceptibility and outcome of *C*. *trachomatis* infections. However, *C. trachomatis* IgG-positive subfertile women with tubal pathology were more than twice as likely to be carriers of the mutant *TLR4* +896 G allele as compared to those without tubal pathology. This observation did not reach statistical significance. In conclusion, both the murine model and the human immunogenetics studies show a slight effect upon *TLR4* deficiency in the severity of infection but not in the susceptibility to infection.
In **Chapter 6**, negative results were found for the -260 C>T SNP in the gene encoding cluster of differentiation (CD) 14.
In **Chapter 7**, subsequently, a carrier trait analysis was performed, since we hypothesized that carrier traits (i.e. carrying multiple SNPs in multiple genes) that likely result in an aberrant immune response are associated with an increased risk of tubal pathology following a *C. trachomatis* infection. In 227 subfertile women, five variations in four genes encoding *C. trachomatis* receptors [TLR4, TLR9, CD14 and caspase recruitment domain (CARD) 15 / nucleotide-binding oligomerization domain (NOD) 2] were studied. The presence of two or more SNPs tended to corre-

late with an increased risk of tubal pathology following a *C. trachomatis* infection (73%) as compared to a lower number of SNPs (33%). An adequate recognition of *C. trachomatis* by receptors in the genital tract seems to be a relevant step in the immune response, and may play a role in protecting the host against the development of late sequelae following a *C. trachomatis* infection.

Persistent *C. trachomatis* infections do not only result in tubal pathology, but have also been associated with impaired implantation capacities due to persistent low-grade endometritis. **Chapter 8** comprised a pilot study using immunohistochemical staining methods and polymerase chain reaction (PCR) to detect *C. trachomatis* in the endometrium of subfertile women. In 40 subfertile women, no correlation was found between immunohistochemical staining results, CAT and tubal status. PCR results were negative in a random subset of endometrial samples (n = 13). Based on findings in literature, further research on this topic should be encouraged.

In **Chapter 9**, the present insight regarding persistent *C. trachomatis* infections and tubal factor subfertility, including the findings as described in this thesis and recommendations for further studies, were discussed.

Samenvatting

Chlamydia (*C.*) *trachomatis* is de meest voorkomende seksueel overdraagbare aandoening in ontwikkelde landen, en treft jaarlijks 35.000 vrouwen in Nederland. Aangezien de meeste vrouwen geen symptomen hebben en dus niet behandeld worden met antibiotica, kunnen *C. trachomatis* infecties opstijgen naar de hogere tractus genitalis en gedurende vele jaren persisteren, waardoor het risico op late complicaties toeneemt. Tot 4.6% van de vrouwen met *C. trachomatis* infecties van de lagere tractus genitalis zal uiteindelijk tubapathologie en subfertiliteit ontwikkelen. De gouden standaard voor het vaststellen van tubapathologie bij subfertiele vrouwen is de laparoscopie met tubatesten. Teneinde niet alle subfertiele vrouwen bloot te stellen aan invasieve tubadiagnostiek, worden screeningstesten — zoals serologische testen of hysterosalpingografie (HSG) — gebruikt om een onderscheid te maken tussen subfertiele vrouwen met een hoog respectievelijk laag risico op tubapathologie.

Hoofdstuk 1 geeft een introductie van het onderwerp en een overzicht van het proefschrift. Het eerste doel van dit proefschrift was om de screeningsstrategie voor het vaststellen van persisterende *C. trachomatis* infecties en tubapathologie bij subfertiele vrouwen te optimaliseren. Het tweede doel was om de immunogenetische bijdrage aan persistentie van *C. trachomatis* infecties te bestuderen. Het derde doel was om verschillende testmethoden voor detectie van persisterende *C. trachomatis* infecties in het endometrium van subfertiele vrouwen te beoordelen.

Persisterende *C. trachomatis* infecties worden beschouwd als een belangrijke risicofactor voor tubapathologie. In **Hoofdstuk 2** en **3** werden diverse serologische markers van persistentie bestudeerd in een cohort van 313 subfertiele vrouwen, die allen een laparoscopie met tubatesten ondergingen om de tubastatus vast te stellen. Naast de momenteel gebruikte screeningstest [*C. trachomatis* immunoglobuline (Ig) antistof test; CAT], werden IgG antistoffen tegen chlamydia heat shock protein 60 (cHSP60) en tegen chlamydia lipopolysaccharide (LPS), IgA antistoffen tegen *C. trachomatis* en het hoog-sensitieve CRP (hs-CRP) gemeten. De prevalentie van tubapathologie — gedefinieerd als uitgebreide adhesies rondom de adnexa en/of distale occlusie van tenminste één tuba bij laparoscopie — was 18.8%. Alle serologische markers van persistentie waren significant vaker aantoonbaar bij vrouwen met tubapathologie ten opzichte van vrouwen zonder tubapathologie. CAT bleef de beste enkele test [odds ratio (OR) 13.9]. Alleen de combinatie CAT (als marker van

een eerdere *C. trachomatis* infectie) en hs-CRP (als marker van persistentie) resulteerde in een significant hogere OR (39.7), en daarom is deze testcombinatie een potentiële screeningsmethode voor in het fertiliteitsonderzoek.
Verder werd de rol van andere *Chlamydiaceae* species in de ontwikkeling van tubapathologie geëvalueerd in **Hoofdstuk 3**. De hypothese was dat *C. pneumoniae* en *C. psittaci* bijdragen aan de pathogenese van tubapathologie door een genus-specifieke auto-immuun ontstekingsrespons teweeg te brengen, maar onze bevindingen konden deze hypothese niet ondersteunen.
In **Hoofdstuk 4** werden drie verschillende screeningsstrategieën (bestaande uit CAT, hs-CRP en HSG) onderzocht in een populatie van 642 subfertiele vrouwen die allen tubatesten (HSG en/of laparoscopie) hadden ondergaan. Het doel van deze observationele studie was om een screeningsstrategie te ontwikkelen die in staat is om op accurate en niet-invasieve wijze het risico op tubapathologie in te schatten. Gebaseerd op onze bevindingen, stelden wij CAT voor als eerste screeningstest voor tubapathologie bij subfertiele vrouwen (risico op tubapathologie respectievelijk 14% bij CAT-negatieve vrouwen en 53% bij CAT-positieve vrouwen). Hs-CRP leek veelbelovend, en de waarde van het HSG bleek beperkt. Wij stelden voor om bij CAT-negatieve vrouwen en vrouwen met normale of inconclusieve HSG's geen laparoscopie meer te verrichten, terwijl de laparoscopie wel verdedigbaar blijft bij CAT-positieve vrouwen of vrouwen met een afwijkend HSG.

Variaties in immunologisch belangrijke gastheergenen worden verondersteld te resulteren in een afwijkende immuunrespons tegen *C. trachomatis* infecties, waardoor het risico op persistentie en tubapathologie toeneemt. **Hoofdstuk 5** beschreef een *toll-like receptor* (*TLR*) *4 knockout* muismodel en een humaan kandidaat-gen model. In *TLR4 knockout* muizen werden *C. trachomatis* infectie en re-infectie langzamer geklaard ten opzichte van *TLR4 wildtype* muizen, wat duidt op een rol van TLR4 in de immuunrespons tegen *C. trachomatis* infecties. Zowel bij 614 vrouwen die een Seksueel Overdraagbare Aandoeningen-polikliniek bezochten als bij 259 subfertiele vrouwen speelde dragerschap van de *TLR4* +896 A>G *single nucleotide polymorphism* (SNP) geen significante rol in vatbaarheid en beloop van *C. trachomatis* infecties. *C. trachomatis* IgG-positieve subfertiele vrouwen met tubapathologie waren twee keer zo vaak drager van het mutante *TLR4* +896 G allel ten opzichte van degenen zonder tubapathologie. Deze bevinding was niet statistisch significant. Concluderend tonen zowel het muismodel als de humane immunogenetische studies aan dat *TLR4* deficiëntie enig effect heeft op de ernst van de infectie, maar niet op de vatbaarheid voor de infectie.
In **Hoofdstuk 6** werden negatieve resultaten gevonden voor de -260 C>T SNP in het gen coderend voor *cluster of differentiation* (CD) 14.
In **Hoofdstuk 7** werd vervolgens een *carrier trait* analyse verricht, aangezien we veronderstelden dat *carrier traits* (dat wil zeggen dragerschap van meerdere SNP's

in meerdere genen), die waarschijnlijk leiden tot een afwijkende immuunrespons, geassocieerd zijn met een verhoogd risico op tubapathologie na een *C. trachomatis* infectie. Vijf variaties in vier genen coderend voor *C. trachomatis* receptoren [TLR4, TLR9, CD14 en *caspase recruitment domain* (CARD) 15 / *nucleotide-binding oligomerization domain* (NOD) 2] werden bestudeerd bij 227 subfertiele vrouwen. De aanwezigheid van twee of meer SNP's leek te correleren met een verhoogd risico op tubapathologie na een *C. trachomatis* infectie (73%) ten opzichte van minder dan twee SNP's (33%). Een adequate herkenning van *C. trachomatis* door receptoren in de tractus genitalis lijkt een belangrijke stap in de immuunrespons te zijn, en zou een rol kunnen spelen bij het beschermen van de gastheer tegen de ontwikkeling van late gevolgen na een *C. trachomatis* infectie.

Persisterende *C. trachomatis* infecties leiden niet alleen tot tubapathologie, maar zijn ook geassocieerd met een verstoord implantatievermogen ten gevolge van een persisterende *low-grade* endometritis. **Hoofdstuk 8** bestaat uit een *pilot* studie, waarbij immunohistochemische kleuringen en *polymerase chain reaction* (PCR) zijn gebruikt om *C. trachomatis* aan te tonen in het endometrium van subfertiele vrouwen. Bij 40 subfertiele vrouwen werd geen correlatie gevonden tussen de resultaten van immunohistochemische kleuringen, CAT en tubastatus. PCR uitslagen waren negatief in een willekeurige subgroep van endometriumbiopten (n = 13). Gebaseerd op bevindingen in de literatuur zou verder onderzoek over dit onderwerp aan te moedigen zijn.

In **Hoofdstuk 9** werden de huidige inzichten in persisterende *C. trachomatis* infecties en subfertiliteit ten gevolge van tubapathologie, inclusief de bevindingen zoals beschreven in dit proefschrift en aanbevelingen voor toekomstig onderzoek, besproken.

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Curriculum vitae

Janneke den Hartog werd op 4 oktober 1976 geboren in Dordrecht. In 1994 behaalde zij het gymnasiumdiploma aan het Johan de Witt-gymnasium te Dordrecht. Van 1994 tot 1995 studeerde zij geneeskunde aan de Katholieke Universiteit Leuven. In 1995 werd gestart met de studie geneeskunde aan de Rijksuniversiteit Limburg te Maastricht. Na het behalen van het artsexamen in augustus 2001 werkte zij van september 2001 tot en met november 2001 als poortarts in het Atrium Medisch Centrum te Heerlen/Brunssum. Van december 2001 tot en met februari 2006 werkte zij als IVF-arts/onderzoeker in het Academisch Ziekenhuis Maastricht. Het onderzoek zoals beschreven in dit proefschrift werd uitgevoerd binnen het onderzoeksinstituut GROW – School for Oncology & Developmental Biology, afdeling Obstetrie en Gynaecologie (promotores: prof. dr. J.L.H. Evers en prof. dr. J.A. Land) en Medische Microbiologie (promotor en co-promotor: prof. dr. C.A. Bruggeman en dr. S.A. Morré). Van maart 2006 tot en met augustus 2006 werkte zij als arts-assistent niet in opleiding op de afdeling Obstetrie en Gynaecologie in het Maaslandziekenhuis te Sittard. Vanaf 16 augustus 2006 is zij in opleiding tot gynaecoloog. De opleiding werd gestart in het Academisch Ziekenhuis Maastricht (opleider: prof. dr. G.G.M. Essed). Per 1 oktober 2008 heeft zij de opleiding voortgezet in het Atrium Medisch Centrum te Heerlen (opleider: dr. F.J.M.E. Roumen).