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# ***CHLAMYDIA TRACHOMATIS AND REPRODUCTIVE HEALTH***

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## **Academic Dissertation**

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*To my family*

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## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

- I. **Rantsi T**, Joki-Korpela P, Wikström E, Öhman H, Bloigu A, Lehtinen M, Gissler M, Tiitinen A, Paavonen J, Surcel H-M. Population based study of prediagnostic antibodies to *Chlamydia trachomatis* in relation to adverse pregnancy outcome. *Sexually Transmitted Diseases*. 2016 Jun;43(6):382–7.
- II. **Rantsi T**, Öhman H, Puolakkainen M, Bloigu A, Paavonen J, Surcel H-M, Tiitinen A, Joki-Korpela P. Predicting tubal factor infertility by using markers of humoral and cell-mediated immune response against *Chlamydia trachomatis*. *American Journal of Reproductive Immunology*. 2018 Nov;80(5): e13051.
- III. **Rantsi T**, Joki-Korpela P, Hokynar K, Kalliala I, Öhman H, Surcel H-M, Paavonen J, Tiitinen A, Puolakkainen M. Serum antibody response to *Chlamydia trachomatis* TroA and HtrA in women with tubal factor infertility. *European Journal of Clinical Microbiology & Infectious Diseases*. 2018 Aug;37(8):1499–1502.
- IV. **Rantsi T**, Joki-Korpela P, Öhman H, Bloigu A, Kalliala I, Puolakkainen M, Paavonen J, Surcel H-M, Tiitinen A. *Chlamydia trachomatis*-induced cell mediated and humoral immune response in women with unexplained infertility. *American Journal of Reproductive Immunology*. 2018 Jul;80(1): e12865.

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## **ABBREVIATIONS**

BV	Bacterial vaginosis
CAT	Chlamydia antibody testing
DNA	Deoxyribonucleic acid
EB	Elementary body
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunoassay
EP	Ectopic pregnancy
FMC	Finnish Maternity Cohort
HPV	Human papillomavirus
HSG	Hysterosalpingography
HSSG	Hysterosalpingosonography
cHSP60	Chlamydial heat shock protein 60 kDa
ICD	International Classification of Diseases
IFN- $\gamma$	Interferon-gamma
IL	Interleukin
IVF	<i>In vitro</i> fertilization
LGV	Lymphogranuloma venereum
LR	Likelihood ratio
MIF	Microimmunofluorescence
MOMP	Major outer membrane protein
NAAT	Nucleic acid amplification test
NIDR	National Infectious Disease Register
NPV	Negative predictive value
OR	Odds ratio
PID	Pelvic inflammatory disease
PCR	Polymerase chain reaction
PPV	Positive predictive value
PROM	Premature rupture of membranes
PTD	Preterm delivery
RB	Reticulate body
RR	Risk ratio
SNP	Single nucleotide polymorphism
STI	Sexually transmitted infection
TFI	Tubal factor infertility
Th	T-helper
TNF- $\alpha$	Tumor necrosis factor-alpha
TOC	Test of cure

## **ABSTRACT**

*Chlamydia trachomatis* infection has been linked to severe reproductive morbidity, including pelvic inflammatory disease (PID), tubal factor infertility (TFI), and ectopic pregnancy (EP). Chlamydial infection has also been associated with miscarriage and preterm delivery (PTD), but the evidence has mostly been based on clinical case-control studies with small study populations, and the data retrieved from population-based studies have been limited.

To further clarify the association between adverse pregnancy outcomes and *C. trachomatis* infection, we performed a seroepidemiologic register and biobank study. We used national population-based health registries to identify the cases with adverse pregnancy outcomes. The cases with EP (n=800) and miscarriage (n=800) were identified through the Finnish Hospital Discharge Register between 1998 and 2005, and cases with PTD (n=1350) were identified from the Finnish Medical Birth Register between 1988 and 2005. The cases were linked to the Finnish Maternity Cohort serum bank to obtain samples for serological analysis. An equal number of women without the outcome diagnosis served as controls. *C. trachomatis* major outer membrane protein (MOMP)-specific IgG antibodies were determined from the serum samples. Our results confirmed the association between serum *C. trachomatis* IgG antibodies and EP at the population level. The seroprevalence rate and the link between antichlamydial antibodies and EP were strongest among women over 35 years of age. We did not find a serological link between *C. trachomatis* infection and miscarriage or PTD.

The link between serum *C. trachomatis* IgG antibodies and TFI has been well established, and chlamydia antibody testing (CAT) has been introduced as a screening test for TFI in the initial infertility workup to select high-risk patients for further tubal evaluation. The persistence of *C. trachomatis* in the upper genital tract has been suggested as one of the key mechanisms in the development of Fallopian tube damage. This persistent form of *C. trachomatis* is featured by the expression of particular proteins, including *C. trachomatis* TroA, HtrA, and 60 kDa chlamydial heat shock protein (cHSP60). Cell-mediated immune response is crucial in the resolution of *C. trachomatis*, but it may also play an important role in the pathogenesis of tubal damage.

*C. trachomatis* may also impair fertility via mechanisms other than occluding the Fallopian tubes, such as causing functional tubal damage or inflammation in the endometrium. It has been suggested that women with *C. trachomatis* IgG antibodies in serum may have a poorer chance of spontaneous pregnancy than seronegative women, even when the tubes are patent. The role of *C. trachomatis*-induced cell-mediated immune response in unexplained infertility is not known.



We studied the role of *C. trachomatis* infection in subfertility by measuring *C. trachomatis*-specific immune responses in a cohort of subfertile women (n=258). Our aim was to develop a specific and sensitive non-invasive test for the prediction of *C. trachomatis*-related TFI. Serum *C. trachomatis*-specific IgG antibody responses were studied using *C. trachomatis* MOMP, cHSP60, and *C. trachomatis* TroA and HtrA as antigens. Cell-mediated immune response was analyzed by an *in vitro* lymphocyte proliferation test using the *C. trachomatis* elementary body (EB) and recombinant cHSP60 as lymphocyte-stimulating antigens. Women with unexplained infertility (n=96) comprised a subcohort. Clinical data on the results of infertility investigations and the outcomes of infertility treatment were prospectively collected from the patient registries of Helsinki University Hospital for 2007–2014.

According to our results, the accuracy of *C. trachomatis* serology in evaluating TFI among an unselected population of subfertile women can be improved by combining serum *C. trachomatis* MOMP and cHSP60 IgG antibody tests or combining markers of *C. trachomatis*-induced cell-mediated and humoral immune responses. Serum antibodies to *C. trachomatis* TroA and HtrA were more common in women with TFI than in women with other causes of subfertility. *C. trachomatis* TroA and HtrA serology have the potential to be further developed into a novel biomarker to predict *C. trachomatis*-related tubal pathology.

Cell-mediated immune response against *C. trachomatis* was common in subfertile women, but neither humoral nor cell-mediated immune response to *C. trachomatis* were associated with unexplained infertility. The presence of serum antichlamydial IgG antibodies was linked to a prolonged time for spontaneous pregnancy in women with unexplained infertility, but pregnancy outcomes, including live birth rate, did not differ between seropositive and seronegative women.

Studies estimating the risk of long-term sequelae following *C. trachomatis* infection are important, because women diagnosed with chlamydial infection are usually worried and need counseling for their future fertility. Our study, together with other population-based data, suggests that the long-term risks following chlamydial infection are lower than previously thought. Since the development of TFI is multifactorial, *C. trachomatis* immune markers in TFI prediction are only of modest value. The risk of reproductive sequelae is higher after recurrent chlamydial infection, and preventive strategies should be planned to recognize the core group at the highest risk for repeat infection.

## FINNISH SUMMARY

*Chlamydia trachomatis* aiheuttama klamydiainfektio on liitetty hankaliin lisääntymisterveyden ongelmiin, kuten sisäsynnytintulehdukseen, munanjohdinperäiseen lapsettomuuteen ja kohdun ulkopuoliseen raskauteen. Klamydiainfektion on ajateltu lisäävän keskenmenon tai ennenaikaisen synnytyksen riskiä, mutta aiemmat tutkimustulokset ovat pohjautuneet pieniin tapaus-verrokkitutkimuksiin ja väestötason tutkimuksia on ollut vähän.

Valtaosalle klamydiainfektion saaneista kehittyy *C. trachomatis* spesifinen vasta-aine- ja soluvälitteinen immuunivaste. Soluvälitteinen immuunivaste on tärkeä klamydiainfektion paranemisessa, mutta sen tiedetään olevan osallisena myös kudosaivurioiden kehittymisessä. Seerumin klamydiaspesifiset vasta-aineet ovat yleisiä munanjohdinperäistä lapsettomuutta sairastavilla naisilla ja klamydiavasta-aineiden mittaamista onkin esitetty osaksi lapsettomuuden alkututkimuksia munanjohdinaurion osoittamiseksi. Ongelmana vasta-ainetestissä on väärrien positiivisten testitulosten suuri määrä, sillä seerumista mitattavissa olevat vasta-aineet kertovat yksilön aiemman altistuksen bakteerille, mutta ei infektion toistumisesta tai kroonistumisesta.

Klamydiainfektio voi aiheuttaa alentunutta hedelmällisyyttä myös muilla mekanismeilla, kuin arpeuttamalla munanjohtimet. Infektio voi vaurioittaa munanjohtimien toimintaa tai aiheuttaa kroonisen kohdun limakalvon tulehduksen johtaen selittämättömään lapsettomuuteen. On todettu, että *C. trachomatis* seropositiivisilla naisilla spontaanin raskauden mahdollisuus on pienempi kuin seronegatiivisilla, vaikka munanjohtimet olisivat avoimet.

Tämän väitöskirjatyon tavoitteena oli tutkia klamydiainfektion merkitystä naisen lisääntymisterveydelle. Rekisteripohjaisessa tutkimuksessa arvioimme väestötasolla sairastetun klamydiainfektion yhteyttä kohdun ulkopuoliseen raskauteen (n=800), keskenmenoon (n=800) ja ennenaikaiseen synnytykseen (n=1350) tutkimalla klamydiaspesifisten IgG-luokan vasta-aineiden esiintyvyyttä näissä tautiryhmissä. Poimimme tapaukset Terveystieteiden ja hyvinvoinnin laitoksen (THL) ylläpitämistä kansallisista rekistereistä (Hoitoilmoitusrekisteri ja Syntymärekisteri) ja yhdistimme tapaukset Äitiseerumipankkiin (Finnish Maternity Cohort, FMC). Seeruminäytteistä määritimme IgG vasta-aineita *C. trachomatis* major outer membrane proteiinia (MOMP) vastaan entsyymi-immunologisella (EIA) -menetelmällä. Tutkimuksemme vahvisti klamydiainfektion ja kohdun ulkopuolisen raskauden yhteyden, vaikkakin *C. trachomatis* vasta-aineiden esiintyvyys olikin matalampi, mitä aikaisemmissa tutkimuksissa on todettu. Vasta-aineprevalenssi nousi naisen iän mukana ollen korkein yli 35-vuotiaiden naisten ryhmässä. Klamydiainfektion ja keskenmenojen tai ennenaikaisen synnytyksen välillä emme todenneet serologista yhteyttä.



## **INTRODUCTION**

*Chlamydia trachomatis* is the most common bacterial sexually transmitted infection (STI) worldwide, with over 130 million new infections occurring annually (World Health Organization, 2017). The highest prevalence of *C. trachomatis* infection is seen in young, sexually active women, aged 20–25 years (National Institute for Health and Welfare, 2017). *C. trachomatis* infection is typically asymptomatic, which enables the effective transmission of the pathogen in the population (Stamm, 1999).

Every sixth couple faces unwilling infertility during their lifetime. Infertility is not only a personal tragedy for couples but also a growing public health issue with declining birth rates in high-income countries. Infertility can be caused by several factors, including male factors, hormonal factors, ovulatory disorder, endometriosis, or tubal factor infertility (TFI). In one-third of infertile couples, the etiology of infertility remains unexplained (Smith, 2003). Untreated *C. trachomatis* infection may ascend from the cervix to the upper genital tract, causing pelvic inflammatory disease (PID) and a risk of TFI. It is possible that *C. trachomatis* may also impair fertility by mechanisms other than occluding the Fallopian tubes, such as causing chronic endometritis and leading to impaired implantation (Coppus *et al.*, 2011). Although *C. trachomatis* infection is strongly associated with TFI, the impact of *C. trachomatis* infection on pregnancy rates in subfertile women with unexplained infertility is unclear.

*C. trachomatis* lower genital tract infection is usually resolved without long-term sequelae, but some women are more susceptible to late sequelae than other. Repeat infections especially are shown to increase the risk of tubal scarring (Davies *et al.*, 2016, Weström *et al.*, 1992). The reproductive sequelae of *C. trachomatis* infection may become apparent several years after the initial infection when the affected woman is trying to become pregnant. *C. trachomatis* infection has also been linked to adverse pregnancy outcomes, including ectopic pregnancy (EP), miscarriage, and preterm delivery (PTD) (Paavonen and Eggert-Kruse, 1999).

Since the link between *C. trachomatis* infection and TFI has been well established in serological studies, serum chlamydia antibody testing (CAT) has been introduced as a TFI screening test in the initial infertility workup (Land and Evers, 2002). With a negative CAT result, further unnecessary or invasive examinations could be avoided, whereas in CAT-positive women, further tubal evaluation could be performed early. The performance of routinely used CAT in TFI prediction is limited because many women have serum antichlamydial antibodies as a marker of previous exposure to the pathogen, but no tubal pathology.

The ability of *C. trachomatis* to morph into a persistent form has been hypothesized as one of the key pathogenetic mechanisms behind tissue damage and reproductive pathologies.

This persistent form has been characterized by the specific transcriptional profile, including the enhanced expression of *C. trachomatis* TroA, HtrA, and 60 kDa chlamydial heat shock (cHSP60) proteins. It has been suggested that serum IgG antibodies to cHSP60 predict TFI more accurately than CAT (Tiitinen *et al.*, 2006), but the results have been controversial (den Hartog *et al.*, 2005, Huston *et al.*, 2010). The presence of serum IgG antibodies to *C. trachomatis* TroA and HtrA has been suggested to indicate upper genital tract chlamydial infection rather than uncomplicated lower genital tract infection (Hokynar *et al.*, 2017).

A cell-mediated immune response to *C. trachomatis* is crucial in the resolution of the pathogen, but it may also contribute to the immunopathological processes, resulting in tubal scarring (Loomis and Starnbach, 2002). *C. trachomatis*-specific cell-mediated immune response has been detected more often in women with TFI than in healthy controls (Öhman *et al.*, 2006, Tiitinen *et al.*, 2006). It has been suggested that combining the markers of *C. trachomatis*-induced cell-mediated immune response to antibody response improves the accuracy of CAT in TFI prediction (Tiitinen *et al.*, 2006).

The aim of this thesis was to evaluate the population for risk of adverse pregnancy outcomes, including EP, miscarriage, and PTD following *C. trachomatis* infection. Another aim was to clarify the impact of *C. trachomatis* infection on reproductive morbidity by measuring *C. trachomatis*-specific cell-mediated and humoral immune responses in a cohort of subfertile women, especially in those with unexplained infertility. We were also aiming to develop a non-invasive test for TFI prediction to simplify the infertility workup.

# **REVIEW OF THE LITERATURE**

## **1. *Chlamydia trachomatis***

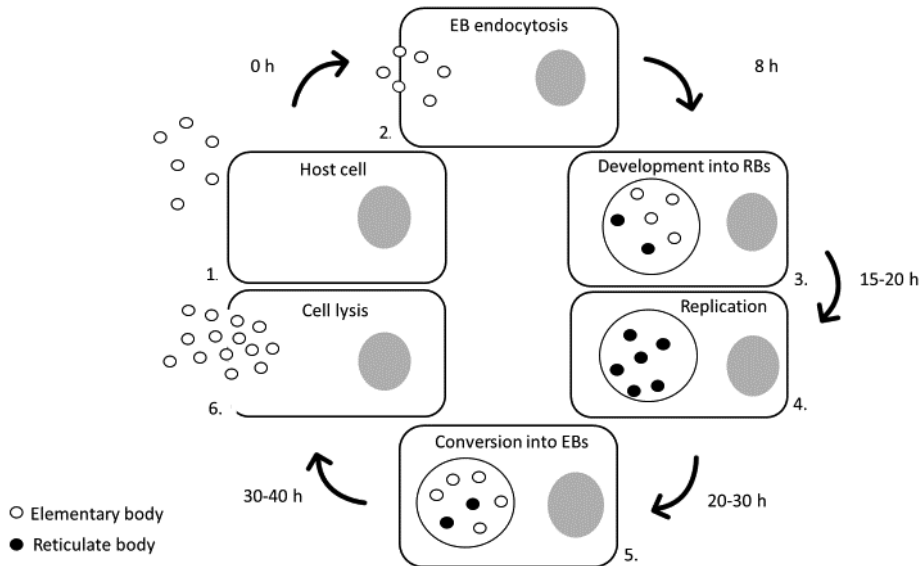
### **1.1. Historical landmarks**

*Chlamydia trachomatis* is a gram-negative bacterium that was first isolated from the female genital tract in 1959 (Jones *et al.*, 1959). The etiologic role of this pathogen in genital infections was revealed in the late 1960s and early 1970s. By the late 1970s, a wide spectrum of the clinical manifestations of chlamydial infection was recognized, and the association of *C. trachomatis* and pelvic inflammatory disease (PID) was found (Paavonen *et al.*, 1979). The culture technique to isolate *C. trachomatis* was developed in 1965, but development of the polymerase chain reaction (PCR) technique in the 1980s started a new era regarding the diagnostics and research of chlamydial infections (Mullis and Faloona, 1987). The first study linking past genital *C. trachomatis* infection to tubal factor infertility (TFI) was published in 1979 (Punnonen *et al.* 1979). Since then, the pathogenesis of *C. trachomatis*–associated tubal pathology has been intensively researched, but the mechanisms are still not yet fully understood.

### **1.2. Developmental cycle**

*C. trachomatis* is an intracellular bacterium that needs living cells to reproduce. The biphasic developmental cycle of *C. trachomatis* is unique, comprised of two functionally and morphologically different forms (Wyrick, 2010) (Figure 1). The extracellular forms, elementary bodies (EBs), are infectious and metabolically inactive forms of the bacteria. After being endocytosed by the host cell, EBs cluster into intracytosolic vacuoles and start transforming into non-infectious, metabolically active reticulate bodies (RBs). RBs utilize nutrients of the host cell and replicate by binary fission. Approximately 1–2 days after host cell infection, RBs start to convert back to infectious EBs, which are released from the cell to breed the infectious process further.

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



### 1.3. *C. trachomatis* serotypes and genome

There are over 20 distinct *C. trachomatis* serotypes identified, of which serotypes A–C are associated with trachoma (Hu *et al.*, 2010), D–K with urogenital infections (Millman *et al.*, 2004, Morr  *et al.*, 1998), and L1–L3 with lymphogranuloma venereum (LGV) infection (Mabey and Peeling, 2002). This distribution is based on an antigenic variation in the chlamydial major outer membrane protein (MOMP) encoded by *ompA*. MOMP is a highly immunogenic protein that accounts for approximately 60% of the mass of the outer membrane of *C. trachomatis* EB (Hatch *et al.*, 1981).

Altogether, 11 *C. trachomatis* serotypes have been isolated from the genital tract. Distinct serotypes have been associated with specific clinical symptoms (Dean *et al.*, 1995), but not all studies have found such a link (Geisler *et al.*, 2003, Millman *et al.*, 2006, Morr  *et al.*, 2000). Serotypes have been suggested to differ in their immunopathogenicity and sensitivity to the host's immune response (Anttila *et al.*, 2001, Byrne, 2010), as well as in the duration of chlamydial infection (Geisler *et al.*, 2008). The most common urogenital serotypes (D, E, and F) are probably the least immunogenic and are linked with asymptomatic infection, which enables them to spread in the population (Gao *et al.*, 2007, Menon *et al.*, 2015).

Several virulence-associated genes have been characterized in a relatively small genome of *C. trachomatis*. Chlamydial chromosomes consist of approximately one million base pairs, being able to encode at least 600 proteins (Stephens *et al.*, 1998). *C. trachomatis* has an extrachromosomal cryptic plasmid that is commonly used as a target sequence in diagnostic nucleic acid amplification tests (NAATs).

In 2006, Sweden experienced an unexpected drop in *C. trachomatis* notifications. This was caused by a new genetic variant of *C. trachomatis* (nvCT) serotype E, which had a 377 base pair deletion in its cryptic plasmid (Ripa and Nilsson, 2007). This strain was undetectable by commercially used NAATs because the primers did not recognize the mutant plasmid. The prevalence of nvCT in some Swedish counties was as high as 65% but rapidly decreased after the re-establishment of NAATs capable of detecting nvCT. However, despite severe failure in *C. trachomatis* diagnostics for some years, it has only a marginal effect on *C. trachomatis*-associated complication rates (Dahlberg *et al.*, 2018). In Finland, the prevalence of nvCT remained low (0.4%) (Niemi *et al.*, 2011).

## 2. Epidemiology of *C. trachomatis* infection

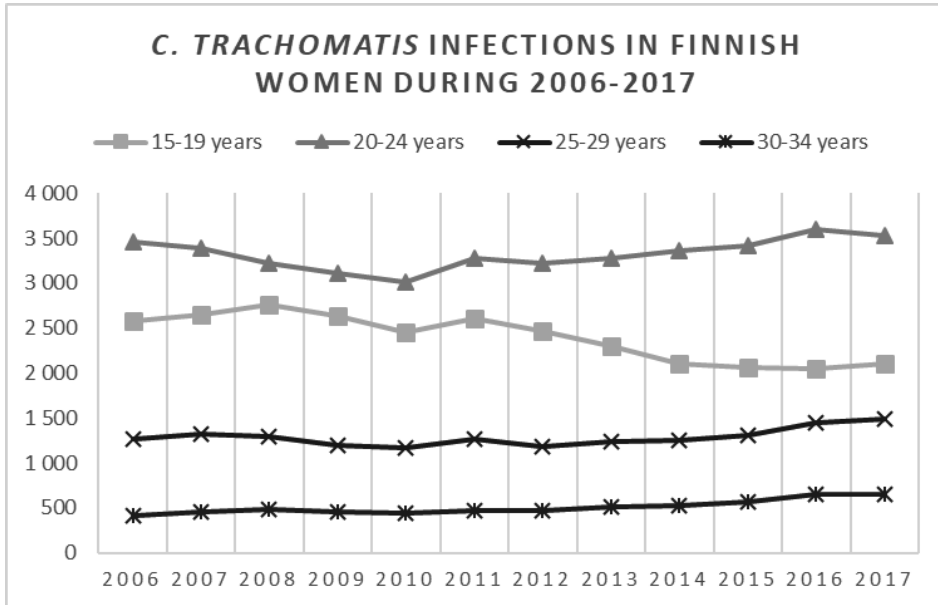
*C. trachomatis* infection is the most common sexually transmitted bacterial infection (STI) worldwide, with over 130 million cases occurring annually (World Health Organization, 2017). There has been an increasing trend in the number of reported infections, but the proportion of *C. trachomatis* notifications that represent a true rise in incidence is unclear (Unemo *et al.*, 2017). High notification rates may reflect the increased number of case finding, rather than a real increase in the incidence (Rekart *et al.*, 2013).

The prevalence of chlamydia ranges from 2% to 17% among asymptomatic women, depending on the study population and country (Bebear and de Barbeyrac, 2009, Wilson *et al.*, 2002). A cumulative incidence of diagnosed chlamydial infections among 31-year-old Finnish women is approximately 11–12% (Karinen *et al.*, 2004). In a recent study from the STI clinic at Helsinki University Hospital, the prevalence of *C. trachomatis* was 6.3% (Hokynar *et al.*, 2018).

Surveillance of *C. trachomatis* infection in Finland is based on cases reported to the National Infectious Disease Register (NIDR), which is maintained by the National Institute for Health and Welfare. Notification in the NIDR includes the personal identification number, gender, age, the place of sampling, and the testing method. In 2017, there were 14 462 *C. trachomatis* infections reported to the NIDR (National Institute for Health and Welfare, 2017). The highest prevalence of *C. trachomatis* infection in Finland is seen in women aged 20–24 years (Figure 2).



**Figure 2.** Rate of reported *C. trachomatis* infections among Finnish women by age in 2006–2017 (National Institute for Health and Welfare, 2017).



## 2.1. Risk factors

Several risk factors have been recognized for chlamydial infection. Chlamydia is associated with sexual risk-taking behavior, such as multiple sexual partners, young age at sexual debut, recent change of partner, and inconsistent condom use (Aghaizu *et al.*, 2014, Harder *et al.*, 2016, Hiltunen-Back *et al.*, 2001). Women have 3.5 times higher risk of *C. trachomatis* infection than men (Miller *et al.*, 2004). Other risk factors for *C. trachomatis* infection include non-white ethnicity and low education level, which has also been linked to *C. trachomatis* infection after adjusting the confounding factors (Harder *et al.*, 2016).

Patients with previously diagnosed chlamydia or another STI are at increased risk of acquiring chlamydia (Miller *et al.*, 2004). Furthermore, vaginal and cervical coinfections have been associated with higher susceptibility to chlamydial infection. For example, women with high-risk human papillomavirus (hrHPV) infection have an increased risk for *C. trachomatis* infection (Aghaizu *et al.*, 2014, Harder *et al.*, 2016). This association has also been observed after controlling high-risk sexual behavior, suggesting that hrHPV may facilitate *C. trachomatis* acquisition through a suppressive effect on the local immune system. Another explanation may be that some shared microbiological or immunological features make these women susceptible to both infections. This hypothesis is supported by the fact that having chlamydial infection may also increase the risk of HPV acquisition,

persistence, and progression to high-grade cervical lesions (Karim *et al.*, 2018, Lehtinen *et al.*, 2011). Similarly, chlamydial infection is found to be a cofactor for the transmission of human immunodeficiency virus (HIV) (Rotchford *et al.*, 2000). *Lactobacilli* that colonize healthy vaginal flora prevent the growth of sexually transmitted pathogens, such as *C. trachomatis* (Mastromarino *et al.*, 2014a, Nardini *et al.*, 2016). Bacterial vaginosis (BV), resulting from *Lactobacilli* being replaced by anaerobic or facultative aerobic bacteria, has been linked to the susceptibility of *C. trachomatis* infection (Wiesenfeld *et al.*, 2003).

## 2.2. Repeat infection

Repeat *C. trachomatis* infections are common, accounting for a remarkable proportion of incident infections (Wikström *et al.*, 2012). Among young adolescents, repeat infection rates of approximately 30% have been reported (Batteiger *et al.*, 2010). In a population-based study from Finland, 34.1% of repeat diagnosis occurred within 12 months (Wikström *et al.*, 2012). Since the failure of antibiotic therapy is unusual, most repeat infections result from a reinfection from an untreated existing partner or a new infected partner (Batteiger *et al.*, 2010). A high proportion of women are reinfected within a short time, which highlights the importance of effective partner treatment and repeat testing (Walker *et al.*, 2012).

## 2.3. Screening

Benefits of screening for *C. trachomatis* infection in high-risk individuals have been supported by many studies (Haggerty *et al.*, 2010, Scholes *et al.*, 1996, Wiesenfeld *et al.*, 2012). The primary aim of screening is detecting and treating asymptomatic infections to prevent late reproductive complications (Land *et al.*, 2010). The secondary aim is reducing the transmission of the pathogen in the population to decrease the overall prevalence of infection. Many national guidelines recommend annual screening of young, sexually active women (Land *et al.*, 2010), but there is a major variation in screening activity among countries (Low *et al.*, 2009).

In opportunistic screening, individuals visiting health professionals are offered a screening test, which is supposed to be repeated at regular intervals. Thus, individuals who do not access health services have no opportunity for testing and some cases of chlamydia may remain undiagnosed. In Finland, there is no national screening program for chlamydia, but the Ministry of Social Affairs and Health (STM) recommends opportunistic screening for all women seeking medical abortion, young (<25 years of age) women seeking contraception, women and men with new sexual partners, a history of genital *C. trachomatis* infection, or another STI. Retesting should be offered annually if the individual has had an earlier *C. trachomatis* infection (STM, 2007).

Chlamydia screening programs are based on assumptions about the natural course of *C. trachomatis* infection, particularly in developing infertility (Gottlieb *et al.*, 2010). However, the long-term and economic impacts of screening have been questioned (Land *et al.*, 2010, Oakeshott *et al.*, 2010, van Valkengoed *et al.*, 2004). Mathematical models and epidemiological studies of *C. trachomatis* transmission and progression have suggested that some complication rates may have been overestimated (Low *et al.*, 2006, Price *et al.*, 2013).

### **3. Clinical manifestations of *C. trachomatis* infection**

#### **3.1. Genitourinary infection in women**

The primary target cells of *C. trachomatis* are columnar epithelial cells of the cervix and urethra. Up to 70–80% of infected women are asymptomatic (Stamm, 1999). Symptoms of lower genital tract infection appear after an incubation period of 7–21 days and may include dysuria, abnormal vaginal discharge, and postcoital bleeding. Chlamydial urethritis may cause leucocytosis in urine, despite a negative culture (Bebear and de Barbeyrac, 2009). Typical local signs of chlamydial cervicitis in speculum examination include mucopurulent vaginal discharge, bleeding from the cervix, and hypertrophic cervical ectopy.

#### **3.2. Pelvic inflammatory disease**

If the cervical *C. trachomatis* infection is not cleared adequately, it may ascend to the upper genital tract, leading to pelvic inflammatory disease (PID). PID has a wide range of clinical manifestations: Some women have silent ascension of infection to the upper genital tract, leading to subclinical PID, while others have severe pelvic infection with chlamydial perihepatitis. Chlamydial PID typically produces mild symptoms but may lead to severe tubal disease (Eschenbach *et al.*, 1997). Subclinical PID has an etiology similar to acute PID and may be twice as common as acute disease (Brunham *et al.*, 2015). Most women with TFI do not report any history of PID, suggesting that inflammation in the upper genital tract can also occur in the absence of clinical signs and symptoms (Wiesenfeld *et al.*, 2005).

It has been estimated that untreated chlamydial infection develops PID in about 10–15% of cases within one year (Oakeshott *et al.*, 2010, Price *et al.*, 2013). Even after ascending to the upper genital tract, single *C. trachomatis* PID is often cleared without reproductive sequelae (Gottlieb *et al.*, 2010).

PID can also be caused by STIs other than chlamydia, or by opportunistic microbes colonizing the female genital tract (Haggerty *et al.*, 2016). Chlamydia has been linked to

approximately 30% of acute PID cases, but the proportion of PID attributable to *C. trachomatis* seems to be declining (Burnett *et al.*, 2012, Goller *et al.*, 2016). However, of all pathogens associated with PID, *C. trachomatis* has been the most widely studied and is most likely linked to infertility.

### **3.3. *C. trachomatis* infection during pregnancy**

*C. trachomatis* infection during pregnancy has been associated with many adverse outcomes for mother and newborn. These include the premature rupture of membranes (PROM), miscarriage, preterm delivery (PTD), stillbirth, and low birth weight of infant (Howie *et al.*, 2011). However, the literature linking *C. trachomatis* infection and adverse obstetric outcomes is inconsistent.

A high proportion of the cases with spontaneous PTD and PROM are associated with ascending genital tract infection (Nadeau *et al.*, 2016). It has been suggested that *C. trachomatis* infection during pregnancy is a significant cause of subsequent PTD (Andrews *et al.*, 2000, Karinen *et al.*, 2005, Rours *et al.*, 2011). In a population-based retrospective study from Washington State USA, chlamydial infection during pregnancy was associated with PROM (RR 1.50 [95% CI 1.03–2.17]) and PTD (RR 1.46 [95% CI 1.08–1.99]) (Blas *et al.*, 2007). In addition, a serological link between *C. trachomatis*, PTD (Claman *et al.*, 1995, Hollegaard *et al.*, 2007), and stillbirth (Gencay *et al.*, 2000) has been observed. However, some of the case-control studies may have suffered from selection bias and inadequate control of potential confounders, including other genital tract infections and other factors known to impact adverse pregnancy outcomes. In a large population-based study from Australia, no significant association was found between a history of having *C. trachomatis* infection and PTD, low birth weight, or stillbirth (Reekie *et al.*, 2018).

Newborns can be infected with *C. trachomatis* during vaginal delivery from an infected mother (Jain, 1999). The neonatal infection usually manifests as conjunctivitis (Kakar *et al.*, 2010), nasopharyngeal infection, or pneumonia (Rours *et al.*, 2009). A recent population-based study from Finland showed that *C. trachomatis* infection in infants is rare (0.22 per 1000 live births), and the risk of vertical transmission from *C. trachomatis* NAAT-positive mothers to neonates is significantly lower than previously reported, at only 0.8% (Honkila *et al.*, 2017).

Miscarriage is the most frequent complication of pregnancy, occurring in approximately 20% of clinically confirmed pregnancies (Giakoumelou *et al.*, 2016). The impact of chlamydial infection on early pregnancy is unclear. It has been hypothesized that *C. trachomatis* may contribute to pregnancy loss by infecting fetal tissues or by inducing an inflammatory response (Witkin and Ledger, 1992). The prevalence of serum antichlamydial antibodies has been suggested to associate with sporadic miscarriages (Baud *et al.*, 2011) and recurrent pregnancy losses (Witkin and Ledger, 1992). However, not all studies have

confirmed such an association (Eggert-Kruse *et al.*, 2014, Paukku *et al.*, 1999, Sugiura-Ogasawara *et al.*, 2005). It is possible that positive serum *C. trachomatis* antibodies are an indirect marker of risk to a spontaneous miscarriage in certain populations, but the causality cannot be proven.

### **3.4. Genitourinary infection in men**

Men have been studied to report any symptoms of *C. trachomatis* infection more often than women (Miller, 2006). Approximately 50% of male infections are asymptomatic (Peipert, 2003). The most common clinical manifestation of chlamydial infection in men is nongonococcal urethritis, which may include urethral discharge, dysuria, or urethral pruritus (Peipert, 2003). Approximately 1% of men with chlamydial urethritis will have reactive arthritis, and in about one-third of the cases, this disease appears as a triad known as Reiter's syndrome (arthritis, uveitis, and urethritis) (O'Connell and Ferone, 2016). The role of chlamydial infection in chronic prostatitis and male factor infertility is controversial (Eggert-Kruse *et al.*, 1996).

### **3.5. Lymphogranuloma venereum**

LGV is an infection caused by invasive *C. trachomatis* serovars (L1–L3). Typically, LGV is characterized by the development of genital ulcer and inguinal femoral lymphadenopathy (Stoner and Cohen, 2015). In past years, high-income countries have experienced a new coming of this disease with new clinical presentation. In Europe, LGV has emerged as a leading cause of proctocolitis in men who have sex with men (MSM). The symptoms of this condition include rectal ulcerations, bleeding, mucopurulent discharge, and lower abdominal pain (Stoner and Cohen, 2015). Chronic infection can lead to the development of perirectal abscess, fissures, and systemic symptoms, such as fever, weight loss, and fatigue. In addition to proctocolitis, women may also have lesions in the labial area, vagina, and cervix (Mabey and Peeling, 2002). LGV diagnostics is challenging because standardized, validated laboratory assays for clinical use are lacking. Diagnosis of LGV is usually based on clinical findings, detecting *C. trachomatis* from anogenital samples by using a nucleic acid amplification test (NAAT), and excluding other potential etiologies for proctocolitis, lymphadenopathy, or genital ulcers (Stoner and Cohen, 2015).

## 4. Diagnosis of *C. trachomatis* infection

### 4.1. Nucleic acid amplification test

Nucleic acid amplification tests (NAATs) are the gold standard for *C. trachomatis* diagnosis (Puolakkainen *et al.*, 1998). Most NAATs are based on polymerase chain reactions (PCRs). Commercially available NAATs are very sensitive compared to culture or antigen tests. They also show very few false-positive results, with the specificity approaching 100% (Meyer, 2016). *C. trachomatis* DNA can be detected in women by testing first-void urine (FVU) or collecting swab samples from the endocervix or vagina. In men, diagnosis can be made by testing FVU or a urethral swab specimen. A NAAT from a rectal or oropharyngeal swab sample is also recommended for detecting extragenital *C. trachomatis* infections (Centers for Disease Control and Prevention, 2015).

Vaginal swabs are the preferred urogenital specimen type in women because they perform as well as cervical swabs, and it is easy for most women to collect vaginal swabs themselves (Van Der Pol *et al.*, 2013). Home-testing is also an option and is preferred for some women. Providing internet-accessed sexually transmitted infection testing (e-STI testing) for high-risk groups may improve the control and management of STIs (Wilson *et al.*, 2017). E-STI testing for *C. trachomatis* and *N. gonorrhoeae* is already available in many clinics in Finland, including the Finnish Student Health Service (FSHS), city of Vantaa and city of Tampere.

The bacterial load of *C. trachomatis* varies by anatomical site and specimen type (Vodstrcil *et al.*, 2015). In women, the highest load is in cervical and vaginal swabs, and the lowest is in FVU samples. It has been suggested that FVU specimens may fail to detect even up to 10% of infections (Meyer, 2016). Table 1 shows the performance of NAAT for *C. trachomatis* from the samples obtained from the various anatomical sites in women. Among men, the bacterial load is similar between the urethral and urine samples.

**Table 1.** The performance of NAAT for *C. trachomatis* from the samples obtained from the various anatomical sites. All data in the table are adapted from Zakher *et al.* (2014).

Specimen type	Sensitivity (%)	Specificity (%)
Endocervix	86.4–95.8	99.3–100.0
Vaginal swab		
Obtained by a clinician	89.9–93.3	98.8–100.0
Collected by a patient	90.7–97.9	99.0–99.9
First-void urine	84.0–96.1	99.5–100.0

## 4.2. Culture

Culture has a specificity of nearly 100% but a sensitivity of 70% or even less compared to the NAAT (Meyer, 2016). Additionally, *C. trachomatis* isolation in culture is technically demanding and needs a relatively long incubation time (3–7 days). Samples have strict transport requirements in terms of both time and temperature. In addition, cell culture requires the sampling of columnar cells, which may be inconvenient for the patient.

## 4.3. Serology

Measuring serum *C. trachomatis* antibodies is not useful in the diagnosis of acute *C. trachomatis* infection as it cannot distinguish a previous infection from a current one. The presence of IgM antibodies to *C. trachomatis* in serum is an unreliable marker of acute infection since it can take up to one month for antibody titers to rise, and in reinfections, IgM antibodies may not be developed (Black CM, 1997). IgG antibodies may persist for years, even after treatment (Puolakkainen, 1998), but interpretation of a single IgG titer is difficult. Serology can be helpful in the diagnosis of LGV and reactive arthritis (Meyer, 2016). Additionally, serology is a valuable tool in seroepidemiological studies and in infertility workup to predict tubal pathology (den Hartog *et al.*, 2008). The most commonly used serological methods to detect *C. trachomatis* antibodies are the microimmunofluorescence (MIF) and enzyme immunoassay (EIA) tests. The performance of serological methods has limitations, including variable sensitivity, specificity, and cross-reactivity with *Chlamydomphila pneumoniae* (Bax *et al.*, 2003, Morr e *et al.*, 2002).

# 5. Treatment of *C. trachomatis* infection

## 5.1. Antibiotics

Oral administration of either a 1 g single-dose of azithromycin or 100 mg of doxycycline twice daily for 7 days is recommended for the treatment of uncomplicated chlamydial infection (Seksitaudit, K yp  Hoito-suositus, 2018). The medication is free of charge in Finland (Tartuntatautilaki). Partners also need treatment, even if they have no signs or symptoms. Sexual contract tracing is mandatory by infectious disease legislation in Finland (Tartuntatautilaki).

*C. trachomatis* has not developed clinically significant antibiotic resistance. This is probably due to the inert nature of EBs, which have little genetic interaction with other organisms, and metabolically active RBs being sequestered inside the cells. In a meta-analysis by Lau *et al.*, azithromycin and doxycycline were comparable in their efficacies for lower genital

tract chlamydial infection, with cure rates of over 95% with both regimens (Lau and Qureshi, 2002).

Some newer evidence has suggested that treatment failure following azithromycin may account for a considerable proportion of repeat *C. trachomatis* notifications (Horner, 2012). Especially the 1 g single-dose azithromycin was suggested to be too short-lived regarding the complex life cycle of *C. trachomatis* (Horner, 2012). In *in vitro* studies, it has also been observed that a persistent chlamydial form develops more easily in the presence of azithromycin compared to doxycycline (Khosropour *et al.*, 2018, Xue *et al.*, 2017).

In a large clinical trial by Geisler *et al.*, azithromycin was slightly less effective, with occasional treatment failure occurring, compared to doxycycline (97% efficacy for azithromycin and 100% for doxycycline) (Geisler *et al.*, 2015). In some women, a 1 g single-dose azithromycin treatment may not be sufficient to reach adequate serum levels of the medicine to eradicate *C. trachomatis*. Azithromycin is also ineffective for *C. trachomatis* rectal colonization, which may lead to urogenital autoinfection from the rectum in some azithromycin-treated women (Hocking *et al.*, 2015).

In clinical practice, the patient's adherence to single-dose azithromycin therapy may be better than to doxycycline. Additionally, azithromycin is a primary treatment during pregnancy (Jacobson *et al.*, 2001). Treatment of upper genital tract chlamydial infection requires a longer duration of antibiotics and combining metronidazole is recommended (Centers for Disease Control and Prevention, 2015). Doxycycline is the drug of choice in treating LGV, and three weeks of therapy are required due to the invasive nature of LGV infection (Stoner and Cohen, 2015).

## 5.2. Test of cure

*C. trachomatis* infection is usually resolved within 1–2 weeks of starting treatment. During that time, sexual contact should be avoided to prevent reinfection and to minimize disease transmission (Paavonen, 2012). There has been an ongoing debate about the need for a test of cure (TOC) after completion of treatment, and major differences exist in practices between countries. In Finland, TOC is recommended within four weeks of therapy completion. If TOC is performed sooner, false-positive results in NAAT are possible due to the presence of non-viable organisms in the sample, which may lead to overtreatment (Gaydos *et al.*, 1998). According to the international recommendations, retesting within 3–12 months is preferred to find reinfected individuals (Centers for Disease Control and Prevention, 2015, British Association for Sexual Health and HIV, 2015). A recent RCT suggested that the optimal time for TOC would be 8 weeks after the initial diagnosis and treatment (van der Helm *et al.*, 2018). In pregnant women, TOC is recommended 3–4 weeks after completion of therapy due to the potential consequences of infection for mother and neonate (Centers for Disease Control and Prevention, 2015).



## 6. Natural course *C. trachomatis* infection

The natural course of chlamydial infection varies widely between individuals, and most women clear the infection without consequences (Gottlieb *et al.*, 2010). Studies on the clinical course of untreated lower genital tract *C. trachomatis* infection show spontaneous clearance rates of up to 45% in one year (Morré *et al.*, 2002) and 94% in four years (Molano *et al.*, 2005). In a study by Geisler *et al.*, spontaneous resolution was observed in 22% of participants between screening and a median treatment time of 15 days (range 6–47 days) (Geisler *et al.*, 2013). However, in some women, infection persists and ascends to the upper genital tract, increasing the risk of late reproductive sequelae.

### 6.1. Immune response to *C. trachomatis* infection

A host's immune response to *C. trachomatis* is complex (Carey and Beagley, 2010). As an obligate intracellular bacterium, *C. trachomatis* induces both a humoral and cell-mediated immune system. Immune response to *C. trachomatis* can promote pathogen clearance or contribute to the immunopathological processes leading to tissue damage and late reproductive sequelae (Howie *et al.*, 2011).

#### 6.1.1 Innate Immune response

After exposure to *C. trachomatis*, the mucosal barrier of the cervix provides the primary defense. The ability of the pathogen to enter this physiological barrier is influenced by hormones and the local immunological environment, including the prevailing cervicovaginal microbiome (Molenaar *et al.*, 2018). Cell-mediated immune response is triggered within 1–2 days after the host's exposure to *C. trachomatis*. Infected epithelial cells induce the production of chemokines and proinflammatory cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF)- $\alpha$ , IL-6, and IL-8 (Roan and Starnbach, 2008). Chemokines recruit monocytes and neutrophils, which are important in producing interferon (IFN)- $\gamma$  to prevent *C. trachomatis* growth (Tseng and Rank, 1998).

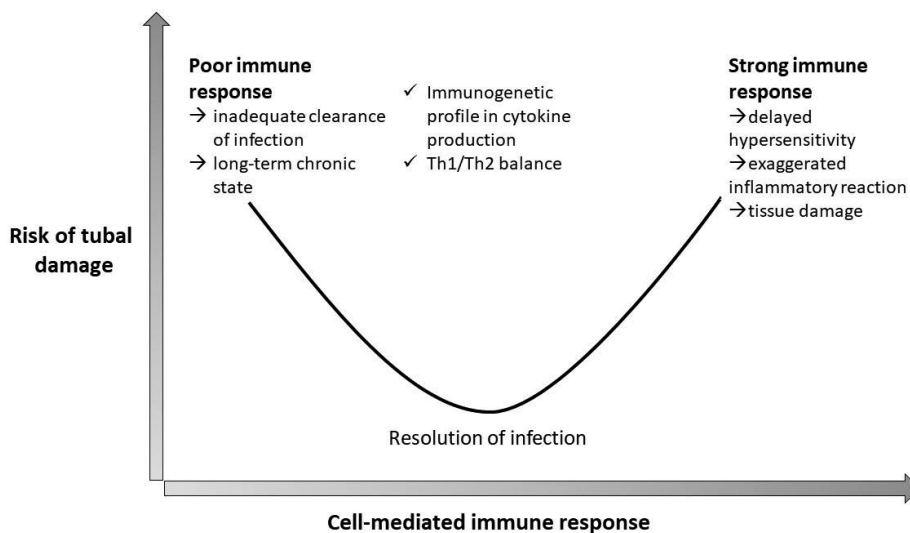
#### 6.1.2 Adaptive immune response

Adaptive immune response is crucial in limiting *C. trachomatis* infection. Both T-cells and B-cells are activated, but T-cells have a more essential role in host defense and clearance of infection (Darville and Hiltke, 2010). One important factor in the cell-mediated immune system is the differentiation of T cells into Th1 and Th2 phenotypes. This classification is based on the distinct functions of these cells and different cytokine profiles, which balance each other's function (Debattista *et al.*, 2003). The proinflammatory and anti-inflammatory

cytokines released from Th1 and Th2 cells mediate opposite effects, regulating the host's defense against *C. trachomatis* infection. Thus, the individual cytokine profile is likely to influence the outcome of infection (Hwang *et al.*, 2015, Öhman *et al.*, 2011). There is a hypothetical balance between the protective and damaging effects of cell-mediated immune response, leading to either the clearing of infection or to tissue pathology due to persistent infection or overstimulated inflammatory response (Figure 3).

In most cases, immune response against *C. trachomatis* is transient and does not develop reproductive sequelae. However, in some women, the inflammatory response persists after clearing the infection, leading delayed immunological hypersensitivity that results in tissue damage and scarring (Menon *et al.*, 2015). During reinfection, T-cells infiltrate to the site of infection more rapidly and in larger numbers than in the primary infection, which strengthens the immunological reaction and can ultimately lead to tissue destruction (Roan and Starnbach, 2008).

**Figure 3.** *The relationship between cell-mediated immune response and risk for tubal damage caused by C. trachomatis. Modified from Debattista et al. (2003).*



The main role of B cells is to produce *C. trachomatis*-specific antibodies, which neutralize the infectivity of the pathogen by the eradication of EBs (Peeling *et al.*, 1984). However, since chlamydial infection is intracellular, antibodies are not crucial in controlling the primary infection. Some infected women do not develop a detectable antibody response

to *C. trachomatis*. In an observational study by Geisler *et al.*, 73% of participants with a current chlamydial infection developed serum IgG antibodies against *C. trachomatis* MOMP (Geisler *et al.*, 2012). In superficial chlamydial infections like cervicitis or urethritis, antibody production may be poorer than in deeper infections (Ngeow, 1996). When induced, serum *C. trachomatis*-specific IgG antibodies are suggested to persist for years after initial infection (Puolakkainen *et al.*, 1986). However, in lower genital tract infection, serum antibody levels may be low, and some initially seropositive individuals may eventually become seronegative as the antibody titers weaken over time in circulation (Horner *et al.*, 2013).

It is well known that younger individuals are more susceptible to chlamydial infection, which has been interpreted as evidence of some protective acquired immunity against *C. trachomatis* (Batteiger *et al.*, 2010). On the contrary, seroprevalence to *C. trachomatis* increases with age (Woodhall *et al.*, 2017). This is supported by a study among sex workers, whose resistance to chlamydial infection correlates with the duration of prostitution (Brunham *et al.*, 1996). However, repeat chlamydial infections are common, indicating that natural immunity to the pathogen is limited and serovar-specific.

Although the incidence of reported chlamydial infections has increased at the population level, the seroprevalence of *C. trachomatis* has declined, indicating that the true burden of infection may have decreased (Lyytikäinen *et al.*, 2008a). This has been explained by the arrested immunity hypothesis, suggesting that early diagnosis and treatment of infection results in an impaired humoral immune response and low production of antibodies (Brunham *et al.*, 2005). Thus, an increased infection rate may reflect the weakened natural immunity that exposes individuals to recurrent chlamydial episodes. It has been observed that in women who resolve the infection spontaneously without antibiotics, the risk of recurrent infection is reduced (Geisler *et al.*, 2013). In addition to the increased susceptibility to reinfections, rapid treatment may also inhibit immune-mediated pathological processes that cause reproductive sequelae (Brunham *et al.*, 2005).

## **6.2. Persistence of *C. trachomatis***

*C. trachomatis* is capable of altering its developmental cycle to generate viable but non-cultivable forms called persistent forms (Beatty *et al.*, 1993). This form is characterized by an altered intracellular morphology accompanied by the formation of enlarged, aberrant cell inclusions and reduced production of infectious chlamydial EBs (Wyrick, 2010). This results in an increased survival of the pathogen and challenges for the host in eradicating infection.

The persistence of *C. trachomatis* can be induced *in vitro* by several factors that favor stressful conditions (Wyrick, 2010). These include the restriction of essential nutrients,

such as amino acids and iron (Raulston *et al.*, 2007), viral coinfection (Raulston *et al.*, 2007), IFN- $\gamma$  (Beatty *et al.*, 1993), and the presence of penicillin (Marsh *et al.*, 2017).

The ability of *C. trachomatis* to transform into a persistent form has been suggested as one of the key pathogenetic mechanisms behind reproductive pathologies. Several *in vitro* (Wyrick, 2010) and animal models (De Clercq *et al.*, 2013) of chlamydial persistence have been developed. From a clinical perspective, persistent infection can be referred to a prolonged exposure to *C. trachomatis*, accompanied by chronic inflammation and incomplete clearance of the pathogen (Schuchardt and Rupp, 2018). However, there is no consensus on the length of this period to produce tissue damage in reproductive organs. Clinical support for the persistence has come from studies where *C. trachomatis* culture-negative women are shown to have *C. trachomatis* DNA in their tubal tissue samples by *in situ* hybridization (Barlow *et al.*, 2001).

### 6.2.1 Serological markers of persistence

Serological markers of persistent *C. trachomatis* infection are of diagnostic value in predicting chlamydia-associated pathologies (Puolakkainen, 2013). Persistence of *C. trachomatis* has been characterized by an altered gene transcription profile and expression of highly immunogenic, specific proteins (Wyrick, 2010). These include chlamydial heat shock protein 60 (cHSP60), chlamydial TroA, and HtrA (Witkin *et al.*, 2017). Heat shock proteins (HSPs) are well-conserved proteins present in all procaryotic and eucaryotic cells. They are essential in different cellular functions, including functioning as chaperones during intracellular folding, as well as assembling and translocating newly synthesized or damaged proteins. The expression of HSPs increases during variable forms of stress, such as infection, inflammation, and exposure to damaging environmental factors (Neuer *et al.*, 2000). In the persistent state of *C. trachomatis*, cHSP60 genes are upregulated, which leads to enhanced expression of cHSP60 (Witkin *et al.*, 2017). CHSP60 is a highly immunogenic antigen for both the humoral and cell-mediated immune system and may play role in the pathogenesis of *C. trachomatis*-induced tissue damage (Kinnunen *et al.*, 2002). Elevated levels of cHSP60-specific serum IgG antibodies have been associated with PID (Peeling *et al.*, 1997) and TFI (Toye *et al.*, 1993).

Human HSP60 (hHSP60), which is one of the first proteins synthesized after fertilization by the epithelial cells of the decidua, shares a 50% amino acid sequence homology with cHSP60. Thus, long-term exposure to cHSP60 in persistent chlamydial infection may lead to the development of autoantibodies against hHSP60 and immunological rejection of the embryo, resulting in early pregnancy loss (Witkin, 2002, Linhares and Witkin, 2010).

Other proteins expressed during persistent chlamydial infection are *C. trachomatis* TroA and HtrA. TroA is a substrate-binding protein in the iron-transport system of *C. trachomatis* and expressed during iron restriction (Miller *et al.*, 2009). High temperature requirement

protein (HtrA) is an important stress response protease and is crucial for the virulence in many intracellular bacteria (Huston *et al.*, 2007). In *C. trachomatis*, HtrA acts as a molecular chaperone to protect the bacterium from stressful conditions and has an essential role during *C. trachomatis* replication (Huston *et al.*, 2007). The levels of TroA and HtrA have been shown to increase under conditions favoring persistence in cell cultures (Huston *et al.*, 2007, Miller *et al.*, 2009, Wyrick, 2010). IgG antibody responses to *C. trachomatis* TroA and HtrA are more common in patients with ascending and repeat chlamydial infection compared to healthy controls (Hokynar *et al.*, 2017).

### **6.2.1 Host immunogenetic factors**

Genetic variation in the magnitude of immune response has a major impact on the course and outcome of chlamydial infection (den Hartog *et al.*, 2006, Öhman *et al.*, 2011). It has been estimated that the host's genetic background accounts for approximately 40% of the variation in *C. trachomatis* outcome (Bailey *et al.*, 2009). Single nucleotide polymorphisms (SNPs) in immunologically important genes may lead to abnormal immune response (den Hartog *et al.*, 2006, Jansen *et al.*, 2016, Öhman *et al.*, 2011). Individual diversity in genes that participate in cell-mediated immune response and the production of certain cytokines is likely to explain some inter-individual differences in the clinical course of infection. For example, a genetic predisposition to low IL-10 and high TNF- $\alpha$  expression has been associated with a strong inflammatory response and scarring of the Fallopian tubes (Öhman *et al.*, 2009).

### **6.2.2 Virulence factors of the pathogen**

Several studies have evaluated different *C. trachomatis* serovars in relation to the variation in the clinical course of chlamydial infection (Geisler *et al.*, 2003, Persson and Osser, 1993). Spontaneous clearance generally occurs more often in women infected with the most common *C. trachomatis* serovars, E and F, whereas persistent infection may be more frequent with less common serovars (Molano *et al.*, 2005). Recent epidemiological studies have suggested that effective chlamydia control programs and active opportunistic screening may have altered the immunobiology of chlamydial infection and resulted in less virulent *C. trachomatis* strains (Byrne, 2010).

### **6.2.3 Microbial environment**

Co-microbes colonizing the host genital tract contribute to *C. trachomatis* survival and persistence. Concurrent infections with *C. trachomatis* and herpes simplex virus (HSV) are common, and *in vitro* studies suggest that the presence of HSV induce *C. trachomatis* to enter into a persistent form (Deka *et al.*, 2006, Mastromarino *et al.*, 2014b). In addition, *N.*

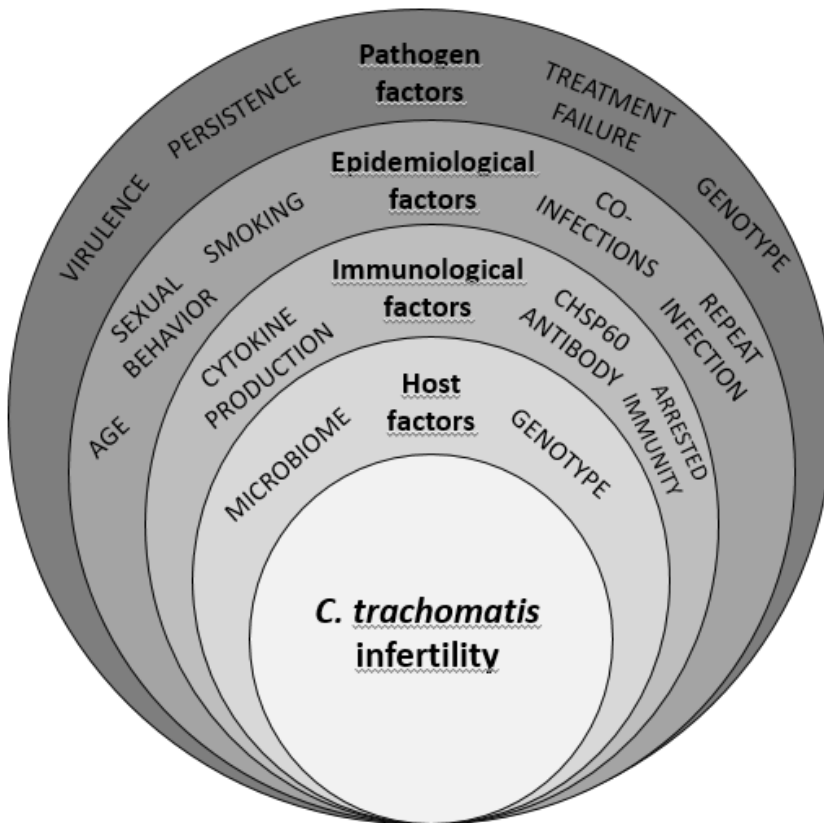
*gonorrhoeae* coinfection may also impair the host immune response to *C. trachomatis* and promote the persistence and ascension of infection to the upper genital tract (Russell *et al.*, 2016).

One critical component of the innate immune response against *C. trachomatis* is interferon- $\gamma$  (IFN- $\gamma$ ) (Aiyar *et al.*, 2014). IFN- $\gamma$  induces the production of indoleamine-2,3-dioxygenase 1 (IDO1), which is the enzyme that degrades tryptophan, an essential amino acid for *C. trachomatis*. As *C. trachomatis* is unable to synthesize tryptophan, production of IDO1 normally leads to the growth restriction and death of the bacterium. However, *C. trachomatis* has developed a mechanism that facilitates its survival despite IFN- $\gamma$ -induced tryptophan depletion. Genital *C. trachomatis* strains have the *trpBA* gene, which enables the synthesis of tryptophan from indole, and obtaining indole from the environment allows *C. trachomatis* survival despite a lack of tryptophan (McClarty *et al.*, 2007). The presence of indole in genital tract secretions depends on the composition of dominating microbes. Indole is not present under *Lactobacilli* dominance, but in shifting the vaginal microbiome towards non-*lactobacilli* dominance and BV, indole can be abundantly detected in vaginal secretions (Witkin *et al.*, 2017). Thus, the prevalence of indole-producing bacteria during BV favors *C. trachomatis* persistence.

## 7. Long-term sequelae of *C. trachomatis* infection

*C. trachomatis* infection can lead to severe reproductive morbidity in women, including ectopic pregnancy (EP) and TFI (Paavonen and Eggert-Kruse, 1999). The pathology resulting from chlamydial infection has been attributed to a severe inflammatory process in the upper genital tract, leading to scarring and loss of the functional tubal epithelium (Mårdh, 2004, Peipert, 2003). The magnitude of the reproductive sequelae risk resulting from chlamydial infection is challenging to estimate (Haggerty *et al.*, 2010). The duration of *C. trachomatis* infection is usually unknown when detected, and once it has been diagnosed, it must be treated. Consequences of infection usually become apparent many years after the initial episode. In prospective studies, follow-up is usually hampered by non-compliance, particularly among women with the highest risk for complications. Because of these aspects concerning studies of the *C. trachomatis* clinical course and sequelae, animal models have played a critical role in investigating *C. trachomatis*-induced immunological mechanisms (De Clercq *et al.*, 2013). A direct link of these studies to reproductive tissue damage in humans is not straightforward because the development of reproductive sequelae is multifactorial, depending on both host and pathogen factors (Figure 4). Linked medical records provide one way of studying morbidity following *C. trachomatis* infection, but it is possible to study only hospitalized diseases and conditions, and the specific diagnostic criteria for reproductive sequelae are usually lacking.

**Figure 4.** Host and pathogen factors that contribute to the development of chlamydial infertility in women. Modified from Menon *et al.* (2015).



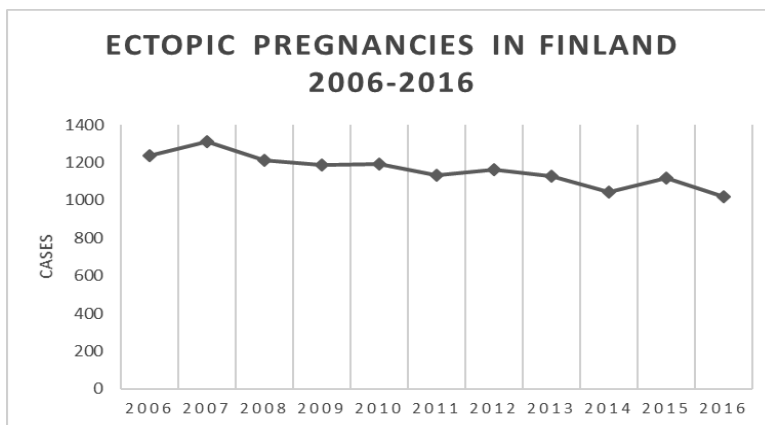
The Uppsala Women’s Cohort study is a large retrospective population-based study evaluating *C. trachomatis*–associated reproductive morbidity (Low *et al.*, 2006). In that study, a cumulative incidence of PID, EP, and infertility after *C. trachomatis* infection was evaluated from health registers. Altogether, 43 715 women aged 15–24 years were followed for five years. They found that the incidence of severe *C. trachomatis*–associated complications at the population level were lower than previously assumed. Similar results were observed in a recent population-based retrospective cohort study from Denmark, where the risk of reproductive complications following *C. trachomatis* infection was studied among over 500 000 women (Davies *et al.*, 2016). According to the results, women with a diagnosed (and assumingly treated) single chlamydial infection had 3.1% cumulative incidence of PID, 2.2% of EP, and 0.6% of TFI. However, repeat *C. trachomatis* infection was still a remarkable risk for reproductive morbidity, increasing PID risk by 30%.

## 7.1. Ectopic pregnancy

EP is the leading cause of pregnancy-related maternal mortality in the first trimester (Barnhart, 2009). The rate of EP has been decreasing in high-income countries, with an incidence of 1–2% of all pregnancies (Bender *et al.*, 2011). Also, in Finland, the overall rate of EP has been slowly declining (Figure 5).

The majority of EPs are located in the Fallopian tube. The pathogenesis of tubal EP is complex and multifactorial, involving subsequent events of chronic inflammation, tubal occlusion or dysfunction, and abnormal implantation (Farquhar, 2005). In a normal pregnancy, gametes and early embryo are transported through the Fallopian tube in an explicit process that requires proper tubal ciliary beating, smooth muscle contraction, and tubal fluid involvement (Shaw *et al.*, 2010). Tubal structure and function can be impaired by pelvic or intratubal adhesions, weakened ciliary beating and smooth muscle contraction, or disturbed early embryo-tubal cell interaction (Shao *et al.*, 2012). The suggested risk factors for EP include previous *C. trachomatis* infection or another STI, previous PID, smoking, *in vitro* fertilization (IVF), and increasing maternal age (Shaw *et al.*, 2010).

**Figure 5.** Incidence of ectopic pregnancies in Finland in 2006–2016 (Hospital Discharge Register, National Institute for Health and Welfare, 2016).



*C. trachomatis* has been considered one of the most important etiological agents behind EP. This has been supported by several studies with different study designs (Haggerty *et al.*, 2010). The link between *C. trachomatis* infection and EP has been found in epidemiological studies (Ankum *et al.*, 1996), and *C. trachomatis* DNA has been found in tubal tissue at the time of EP (Barlow *et al.*, 2001, Brunham *et al.*, 1992b). Furthermore, a



serological link between EP and *C. trachomatis* has been observed (Brunham *et al.*, 1992a, Sziller *et al.*, 1998).

A population-based epidemiological study from Denmark showed that women who tested positive for *C. trachomatis* had a lower rate of EP than those who tested negative, and only 0.7% of women with prior chlamydia infection were estimated to develop EP during their lifetime (Andersen *et al.*, 2005). Furthermore, in the Uppsala Women's Cohort Study, having an EP was associated with low socioeconomic status but not with previous *C. trachomatis* infection (Low *et al.*, 2006). A recent estimation by Price *et al.* suggested that 5% of all EPs are attributable to *C. trachomatis* in women aged 16–44 years (Price *et al.*, 2016).

## 7.2. Tubal factor infertility

TFI can be considered as subfertility due to tuboperitoneal factors (post-infectious tubal damage, tubal occlusion, and pelvic adhesions). TFI accounts for 10–30% of subfertility in high-income countries (Snick *et al.*, 1997, Wiesenfeld *et al.* 2012, Tsevat *et al.* 2017). PID is the major cause of TFI, but most women have never been diagnosed with PID because their infections are asymptomatic or subclinical (Wiesenfeld *et al.*, 2012). Seroepidemiological studies have linked *C. trachomatis* to TFI by showing a strong association between serum antichlamydial antibodies and TFI (Punnonen *et al.*, 1979, Conway *et al.*, 1984, Paavonen and Eggert-Kruse, 1999).

The pathogenesis of *C. trachomatis*–associated TFI is not fully understood (Menon *et al.*, 2015). Data derived from epidemiological studies and animal models suggest that the detrimental effects of *C. trachomatis* are linked to either reinfection or persistent infection (Grayston *et al.*, 1985, Hillis *et al.*, 1997). Ascension of *C. trachomatis* to the upper genital tract, prolonged host exposure to the pathogen, or antigens released by *C. trachomatis* may cause the destruction and scarring of the tubal epithelium, leading to occlusion (Menon *et al.*, 2015). Because the target tissue to *C. trachomatis* is particularly the columnar epithelium, the densely ciliated ampullary segment of the Fallopian tube is most susceptible to *C. trachomatis* infection. The risk of TFI after PID is approximately 9% after one episode, 20% after two episodes, and 40% after three PID episodes (Weström *et al.*, 1992).

The link between *C. trachomatis* infection and infertility has been intensively researched for decades. In women with TFI, *C. trachomatis* DNA has been shown by *in situ* hybridizations from biopsies obtained in laparoscopy (Patton *et al.*, 1994). In mathematical modeling, the proportion of TFI attributable to *C. trachomatis* is estimated to be approximately 45% (Ades *et al.*, 2017). Serological studies have shown serum *C. trachomatis* IgG antibodies in 30–70% of women with tubal pathology (Eggert-Kruse *et al.*,

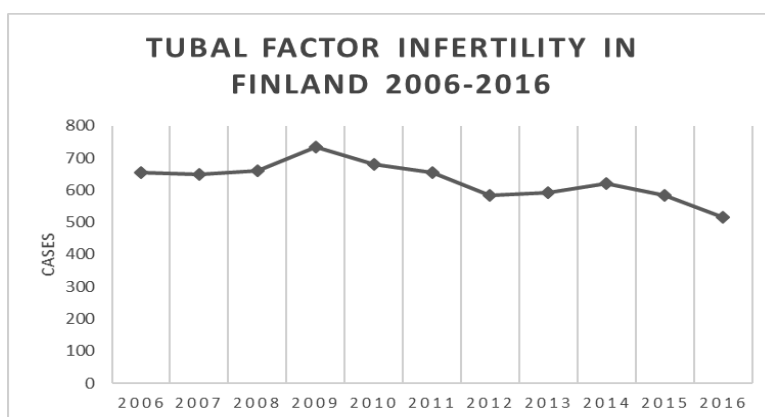
1997, Mouton *et al.*, 2002) compared to 10–20% in the general reproductive-aged female population (Lyytikäinen *et al.*, 2008b).

The proportion of TFI attributable to chlamydial infection is not easy to estimate. TFI can be caused by a variety of factors other than *C. trachomatis* and may appear for a long time after exposure to the pathogen. Additionally, some women may have TFI due to causes not related to chlamydia but to coincidental exposure to *C. trachomatis* as evidenced by positive serum antibodies.

Tubal damage can vary by extent, anatomical location, or nature. Tubal function may be impaired without a visible tubal occlusion (Coppus *et al.*, 2011). The most severe form of TFI is bilateral hydrosalpinges, which are known to predict the lowest chance for natural conception (Akanke, 2007). In those cases, the best outcome results from assisted reproductive technology (ART). TFI management by intrauterine insemination (IUI) in the case of unilateral tubal blockade has been under debate. Women with unilateral tubal pathology often have lower live birth rates (LBR) than subfertile women with patent tubes when treated by IUI, suggesting that IVF might be a better treatment in those cases (Berker *et al.*, 2014, Cochet *et al.*, 2017). However, absolute infertility (sterility) is rare, and many women with TFI usually have a possibility to conceive spontaneously (Hull and Fleming, 1995).

The proportion of TFI associated with ART has decreased in the United States in 2000–2010 (from 26% to 15%) (Kawwass *et al.*, 2013). A similar declining trend of TFI incidence has been observed in Finland (Figure 6). In 2016, the proportion of TFI from all infertility diagnoses was 6.5% (Hospital Discharge Register, National Institute for Health and Welfare, 2016).

**Figure 6.** Incidence of TFI in Finland in 2006–2016 (Hospital Discharge Register, National Institute for Health and Welfare, 2016).



TFI has been linked to several adverse reproductive outcomes, leading to lower LBR compared to women with another cause for subfertility (Camus *et al.*, 1999). In a large retrospective study from the United States, 1.4 million ART cycles were analyzed between 2000 and 2010 using the National ART Surveillance System, and reproductive outcomes were compared between women with TFI and with male factor infertility (Kawwass *et al.*, 2013). Firstly, women with TFI were more likely to have fewer oocytes retrieved and fewer ART cycles resulting in LBR. They also had a slightly higher risk of miscarriage (14.0% vs. 12.7%, RR 1.11 [95% CI 1.04–1.11],  $p < 0.001$ ) and preterm birth (15.8% vs. 11.6%, RR 1.37 [95% CI 1.32–1.43],  $p < 0.001$ ). However, the study population was also likely to include patients with untreated hydrosalpinges, which are known to adversely impact the pregnancy outcome (Camus *et al.*, 1999, Strandell *et al.*, 1999). According to the current guidelines, laparoscopic salpingectomy or proximal tubal ligation are recommended to improve IVF outcomes in women with hydrosalpinges (Dreyer *et al.*, 2016, Kontoravdis *et al.*, 2006).

### **7.2.1 Evaluation of tubal patency**

The gold standard for evaluating tubal status has been laparoscopy with chromopertubation, by which both tubal patency and the presence of peritubal adhesions can be assessed (Mol *et al.*, 2001). Laparoscopy has several disadvantages, however: it is an invasive and expensive procedure requiring general anesthesia, and there is a risk of complications. It is usually indicated for subfertile women who would benefit from the operation, for example in those with large endometrioma requiring operational treatment or if severe symptoms consistent with endometriosis are present (Saunders *et al.*, 2011).

An alternative procedure for tubal evaluation used in many fertility clinics is hysterosalpingography (HSG), the radiographic evaluation of the uterus and Fallopian tubes. It is regarded as an effective screening method for the evaluation of tubal patency and uterine cavity morphology (Simpson *et al.*, 2006). However, it gives no information about the ovaries, and it exposes patients to ionizing radiation and potentially allergenic contrast media.

Hysterosalpingosonography (HSSG) is an ultrasound-based technique in which a mixture of saline and air or specific echogenic contrast media (HyCoSy) is applied to the uterus, and the path of air bubbles or contrast media is followed through the Fallopian tubes. HSSG is considered quick and well-tolerated, so it is feasible in the outpatient setting (Maheux-Lacroix *et al.*, 2014). Adverse side effects of this method include discomfort, pelvic pain, post-procedure vaginal bleeding, vasovagal reaction, and nausea (Dessole *et al.*, 2003). Compared to HSG, HSSG has better sensitivity and specificity in detecting intrauterine pathologies, such as fibromas, polyps, or synechiae (Maheux-Lacroix *et al.*, 2014). It also

allows the concomitant visualization of the ovaries, which may be relevant in relation to subfertility (Saunders *et al.*, 2011).

Both HSG and HSSG include the possibility of false-positive results because of tubal spasm. In a recent meta-analysis (Maheux-Lacroix *et al.*, 2014), HSSG and HSG were compared to laparoscopy; HSSG showed a sensitivity and specificity of 95% (78%–99%) and 93% (89%–96%), and HSG showed 94% (74%–99%) and 92% (87%–95%) in diagnosing tubal occlusion. However, neither method reveals an impaired tubal function. Non-invasive biomarkers for the prediction of *C. trachomatis*-associated TFI have been under development (Land *et al.*, 2003, den Hartog *et al.*, 2005, Menon *et al.*, 2016).

### **7.2.2 C. trachomatis-induced immunological markers in TFI screening**

Serological markers of persistent *C. trachomatis* infection as indicators of female upper genital tract pathology have been widely studied. Serum chlamydial IgG antibody testing (CAT) has been introduced into the infertility workup as an initial TFI screening test to select high-risk patients for laparoscopy (den Hartog *et al.*, 2008, Mol *et al.*, 1997). A negative CAT result may help to avoid further unnecessary or invasive examinations, whereas in CAT-positive women, further examinations can be performed early to avoid long-term expectant management. CAT titers correlate quantitatively to the severity of tubal damage (Akande *et al.*, 2003, El Hakim *et al.*, 2010) and remain detectable in serum for years, even after antibiotic treatment (Gijssen *et al.*, 2002, Puolakkainen *et al.*, 1986).

The evaluation of the diagnostic accuracy of CAT in TFI screening is complicated. The predictive value of CAT depends on the performance of the laboratory assay, the definition of TFI, and the reference group. TFI can be defined as tubal occlusion (bilateral or unilateral), hydrosalpinx, or any peritubal adhesions, which can reduce fecundity even in the absence of tubal occlusion. The serologic assays that are used in *C. trachomatis* serology differ in antigen source. When using a broadly reacting antigen, a cross-reaction between other chlamydia serotypes or species, such as *C. pneumoniae*, is possible (Land *et al.*, 1998). The cut-off value for positive CAT also affects its accuracy in TFI detection, and in clinical practice, the optimal cut-off value depends individually. In some patients, for example in older women who start mild fertility treatment (i.e., ovarian stimulation or IUI), it is important to detect TFI with high reliability. In some patients, on the other hand, it is more important to rule out TFI to avoid unnecessary invasive testing, for example in young women with good prognosis for spontaneous pregnancy.

In any case, the performance of CAT in TFI prediction has limitations mainly due to false-positive cases resulting in low positive predictive value (PPV) (den Hartog *et al.*, 2008). Many women have serum IgG antibodies to *C. trachomatis* without having any tubal pathology or even subfertility. The presence of serum antibodies against *C. trachomatis* indicates only the previous exposure to the pathogen, but it cannot discriminate cleared

infection from persistent infection, which particularly increases the risk of tubal damage. CHSP60 is expressed during persistent *C. trachomatis* infection, and serum antibody response to cHSP60 has been strongly associated with TFI (Ault *et al.*, 1998, Toye *et al.*, 1993). It has been suggested that cHSP60 IgG antibody testing predicts *C. trachomatis*-associated TFI more accurately than CAT (Land and Evers, 2002, Tiitinen *et al.*, 2006), but controversial results have also been reported (den Hartog *et al.*, 2005, Huston *et al.*, 2010). Den Hartog *et al.* compared three immunological markers suggestive of persistent chlamydial infection, *C. trachomatis* IgA, cHSP60 IgG, and high-sensitive C-reactive protein (hs-CRP) as single tests and in combination with CAT (den Hartog *et al.*, 2005). According to their results, the predictive value of CAT could be improved by combining the hs-CRP test with CAT, but *C. trachomatis* IgA or cHSP60 did not increase the accuracy of CAT.

Cell-mediated immune response to *C. trachomatis* is considered crucial in the resolution of chlamydial infection, but it has been suggested to contribute to immunopathological processes, resulting in tubal scarring (Loomis and Starnbach, 2002). Cell-mediated immune response to *C. trachomatis* EB is detected more often in women with TFI than in healthy controls (Öhman *et al.*, 2006, Tiitinen *et al.*, 2006). Similarly, cell-mediated immune response to cHSP60 has been linked to repeat or chronic *C. trachomatis* infection (Kinnunen *et al.*, 2002, Witkin *et al.*, 1994). Combining the markers of *C. trachomatis*-induced cell-mediated immune response with serology has been suggested to improve the accuracy of CAT (Tiitinen *et al.*, 2006).

Since the immunogenetic variation of the host contributes to the immune response against *C. trachomatis* (Öhman *et al.*, 2006), also adding genetic biomarkers to TFI screening has been proposed to result in more accurate tests to identify individuals at low or high risk for tubal pathology (den Hartog *et al.*, 2006, Jansen *et al.*, 2016).

### **7.3. The role of *C. trachomatis* in unexplained infertility**

Infertility is defined as the inability of a sexually active, non-contracepting couple to achieve a pregnancy in 12 months (Zegers-Hochschild *et al.*, 2017). However, as complete infertility is rare, the term subfertility is generally preferred to describe any form of reduced fertility with a prolonged time of unwanted non-conception (Gnoth *et al.*, 2005). Unexplained infertility is diagnosed in approximately 30% of subfertile couples when standard infertility investigations yield normal results (Brandes *et al.*, 2011). However, the diagnosis of unexplained infertility is inaccurate due to the lack of a specific test, and some cases may be misdiagnosed. The most probable causes for misdiagnosis are mild endometriosis, age-related or premature ovarian failure, and immunological causes (Ray *et al.*, 2012). A mild degree of TFI may be classified also as unexplained infertility since the diagnostic methods for tubal evaluation are inaccurate and do not reveal impaired tubal function. Silent PID may also contribute to unexplained infertility, mainly due to chronic

inflammation and local immunological response in the endometrium, impairing the implantation of the embryo (Wiesenfeld *et al.*, 2012).

Couples with unexplained infertility usually have a good prognosis for natural conception, and expectant management is recommended (Brandes *et al.*, 2011). However, the infertility treatment strategy depends on the estimated prognosis of spontaneous pregnancy, in which the age of the female and the duration of infertility play the most important roles (Hunault *et al.*, 2004). Generally, treatment is indicated if the infertility lasts more than two years or if the female is >35 years of age (Bhattacharya *et al.*, 2008). The first line of treatment in unexplained infertility is usually ovarian stimulation with or without IUI, but the effectiveness of this treatment compared to expectant management has been questioned (Veltman-Verhulst *et al.*, 2016). In a recent clinical trial, it was shown that those treated with ovarian stimulation and IUI had significantly higher cumulative LBRs than women who applied for expectant management (31% vs. 9%, RR 3.41 [95% CI 1.71–6.79],  $p=0.003$ ) (Farquhar *et al.*, 2018). IVF is considered the most effective treatment for unexplained infertility, but it is invasive and expensive (Pandian *et al.*, 2015). However, immediate IVF is the best treatment for couples with long durations of subfertility or with older female partners.

The role of *C. trachomatis* in unexplained infertility is not clear. It has been suggested that subfertile women with positive serum *C. trachomatis* antibodies and patent Fallopian tubes may have a lower chance for spontaneous pregnancy than women without antichlamydial antibodies (Coppus *et al.*, 2011, Steiner *et al.*, 2015). These women may have minor tubal damage resulting in impaired tubal function or endometrial inflammation resulting in impaired implantation. In a large, multicentral study from the Netherlands, seropositivity to *C. trachomatis* was associated with a 33% lower probability of an ongoing pregnancy at a 12-month follow-up in women with unexplained infertility (Coppus *et al.*, 2011). In another study (El Hakim *et al.*, 2009), seropositivity to *C. trachomatis* did not adversely affect overall pregnancy rates, but the time to natural conception was not studied there.

#### **7.4. The role of *C. trachomatis* in male factor infertility**

The potential impact of *C. trachomatis* infection on male reproductive health is controversial (Eggert-Kruse *et al.*, 2003, Paavonen and Eggert-Kruse, 1999). The prevalence of serum antichlamydial antibodies has been observed to be higher among men suffering from subfertility than in fertile controls (Idahl *et al.*, 2004, Joki-Korpela *et al.*, 2009). *C. trachomatis* vasitis and epididymitis may be risk factors for obstructive azoospermia through the scarring of the ejaculatory ducts (Gonzales *et al.*, 2004). *C. trachomatis* may also influence male fertility by inducing sperm antibodies (Munoz and Witkin, 1995) or by reducing sperm quality (Idahl *et al.*, 2007, Joki-Korpela *et al.*, 2009). *C. trachomatis* may

directly cause sperm DNA damage, and the high rate of DNA fragmentation index (DFI) in sperm has been associated with male chlamydial infection (Moazenchi *et al.*, 2018).

Furthermore, chlamydial EB can attach to sperm and ascend to the upper female genital tract (Land and Evers, 2002). Prospective studies have demonstrated that the fertility of a subfertile couple is significantly reduced when the male partner has serum *C. trachomatis* antibodies (Eggert-Kruse *et al.*, 1990, Idahl *et al.*, 2004). This finding is not necessarily related to reduced semen quality but is linked to a higher proportion of TFI in their female partners (Eggert-Kruse *et al.*, 1990). In a population-based study from Finland, the presence of serum *C. trachomatis*-specific antibodies in men was even more clearly associated with subfertility than in women (Karinen *et al.*, 2004).

### **7.5. The impact of *C. trachomatis* on the outcome of infertility treatment**

Positive *C. trachomatis* serology has been associated with reduced spontaneous pregnancy rates among subfertile women (Keltz *et al.*, 2013), but the impact of *C. trachomatis* infection on treatment-related pregnancy outcome has been controversial. Serum *C. trachomatis* and cHSP60 antibodies have been associated with poor pregnancy outcomes after IVF (Keay *et al.*, 1998, Licciardi *et al.*, 1992), but not all studies have found such a link (de Barbeyrac *et al.*, 2006, Spandorfer *et al.*, 1999). Local antichlamydial antibodies in cervical secretions have been suggested to result in IVF failure (Witkin *et al.*, 1994). *C. trachomatis* serology has also been studied using the follicular fluid during oocyte retrieval, and a correlation between local *C. trachomatis* antibody response and impaired oocyte maturation has been observed (Neuer *et al.*, 1997, Pacchiarotti *et al.*, 2009). Furthermore, the presence of the cHSP60 antibody in the follicular fluid has been predictive of poor outcomes in IVF treatment (Jakus *et al.*, 2008).

## **AIMS OF THE STUDY**

The aim of this thesis was to study the role of *C. trachomatis* infection in adverse pregnancy outcomes and subfertility by measuring *C. trachomatis*-specific immune responses. Another aim was to develop a specific and sensitive non-invasive test for TFI prediction in subfertile women to simplify infertility workup.

Specific aims of the studies were:

1. To evaluate the link between past *C. trachomatis* infection and ectopic pregnancy, miscarriage, and preterm birth in a population-based longitudinal biobank health registry setting (I).
2. To study whether combining markers of cell-mediated and humoral immune response against *C. trachomatis* improves the accuracy of serology in the prediction of TFI in clinical practice (II).
3. To investigate the humoral immune response against two *C. trachomatis* proteins, TroA and HtrA, expressed during persistent *C. trachomatis* infection in subfertile women (III).
4. To evaluate the impact of past chlamydial infection on pregnancy outcomes in women with unexplained infertility by analyzing both humoral and cell-mediated immune responses against *C. trachomatis* (IV).



# **MATERIALS AND METHODS**

## **1. Study populations**

### **1.1 Population-based register study (Study I)**

We used national population-based health registers to identify cases of ectopic pregnancy (EP), miscarriage, or preterm delivery (PTD). The cases were further linked to the Finnish Maternity Cohort (FMC) serum bank to analyze *C. trachomatis*-specific serum IgG antibodies.

#### **1.1.1. Finnish Maternity Cohort**

The Finnish Maternity Cohort (FMC) was established in 1983, storing serum samples collected from pregnant women for research use. FMC contains over 2 million serum samples, which are collected during early pregnancy for the congenital infection (syphilis, HIV, and hepatitis B) screening. Approximately 99% of pregnant Finnish women participate in the serological screening of congenital infections. Serum samples are drawn during the first antenatal clinic visit (mean  $10.9 \pm 2.9$  gestational weeks) and stored at  $-25^{\circ}\text{C}$  at a central biorepository in Oulu, Finland. The FMC materials were previously maintained by the National Institute for Health and Welfare but were transferred to Biobank Borealis in autumn 2017.

#### **1.1.2. Hospital Discharge Register**

The Hospital Discharge Register (HDR) was established in 1967 and is maintained by the National Institute for Health and Welfare. It contains information on all hospitalizations, including all day surgical procedures since 1994 and all outpatient clinic visits since 1998. HDR data are categorized according to the International Classification of Diseases (ICD) codes and the Finnish version of the Nordic Classification of Surgical Procedures.

#### **1.1.3. Finnish Medical Birth Register**

The Finnish Medical Birth Register (MBR) was established in 1987 and is maintained by the National Institute for Health and Welfare. The MBR contains data on all obstetric outcomes from all birth units in Finland and covers information on all deliveries or stillbirths over 22 gestational weeks and for infants weighing 500 g or more. The quality of the register is excellent, since it covers over 99.9% of all deliveries in Finland.

A total of 69 333 cases with EP or miscarriage were found through the HDR during 1998–2005 according to the International Classification of Disease, 10<sup>th</sup> Revision (ICD-10) codes O00 and O03. A total of 52 434 cases with PTD (<37 gestational weeks) were found from the MBR during 1988–2005. Cases were linked to the FMC by personal identity codes. A total of 1141 cases with EP, 10 394 with miscarriage, and 51 668 with PTD were identified. Altogether, 800 women with EP (100 per year), 800 with miscarriage (100 per year), and 1350 women with PTD (150 per 2 years, or 50 per gestational age group: <28, 28–31+6, 32–36+6) were randomly selected for serological analysis. An equal number of women (n=2950) without the diagnosis served as controls. The cases and controls were matched by sampling time, age at serum sampling, and postal code district.

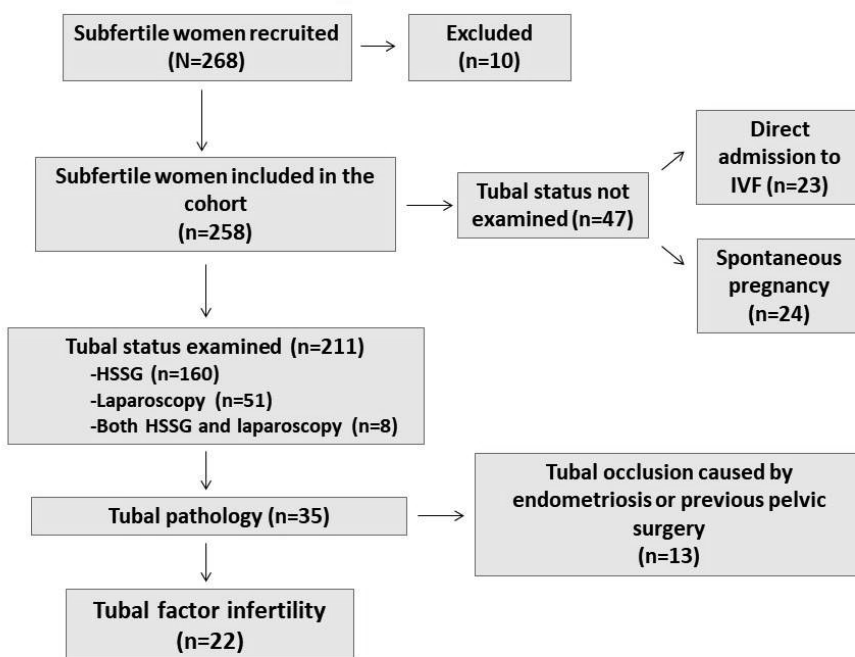
## **1.2 Prospective cohort study (Studies II, III, and IV)**

Altogether, 268 subfertile couples were recruited between July 2007 and December 2010 at the Department of Obstetrics and Gynecology, Helsinki University Hospital, Finland. Infertility workup was performed after at least one year of unprotected intercourse according to the clinic's routine protocol. Gynecological examination including ultrasonography of the uterus and adnexa was performed during the first outpatient clinic visit. Serum gonadotropins, prolactin, and thyroid function tests were analyzed to diagnose hormonal disorders. The ovulatory cycle was confirmed by monitoring follicular growth and measuring mid-luteal serum progesterone. A urogenital swab sample was collected to diagnose *C. trachomatis* by nucleic acid amplification test (NAAT) (Gaydos *et al.*, 2004). Semen samples from male partners were analyzed according to the World Health Organization criteria (Cooper *et al.*, 2010). Peripheral blood samples for immunological analysis were collected at the first outpatient clinic visit, along with the routine samples needed for infertility evaluation.

The final study population (Studies II and III) consisted of 258 subfertile women, of whom 211 underwent tubal examination. Tubal status was primarily evaluated by hysterosalpingosonography (HSSG) in 160 (75.8%) cases. HSSG results were classified as no occlusion, unilateral occlusion, or bilateral occlusion. Bilateral tubal occlusion observed in HSSG was confirmed by laparoscopy, which was performed in 51 (24.2%) cases as a primary method for tubal evaluation. Laparoscopy was also performed in the presence of large endometrioma requiring operational treatment or in cases with severe symptoms of endometriosis. TFI was defined as an occlusion of at least one tube. Women with tubal occlusion due to endometriosis or prior pelvic surgery, besides uncomplicated cesarean section (n=13), were included in the non-TFI group. Tubal examination was not performed in 23 (8.9%) of 258 women who were directly admitted to the planned fertility treatment and in 24 (9.3%) women who conceived spontaneously before tubal evaluation. Cases with a history of EP or EP during the infertility workup were classified as having TFI. Women

with an etiology for subfertility other than TFI served as the reference group. The flowchart of the study population is presented in Figure 7.

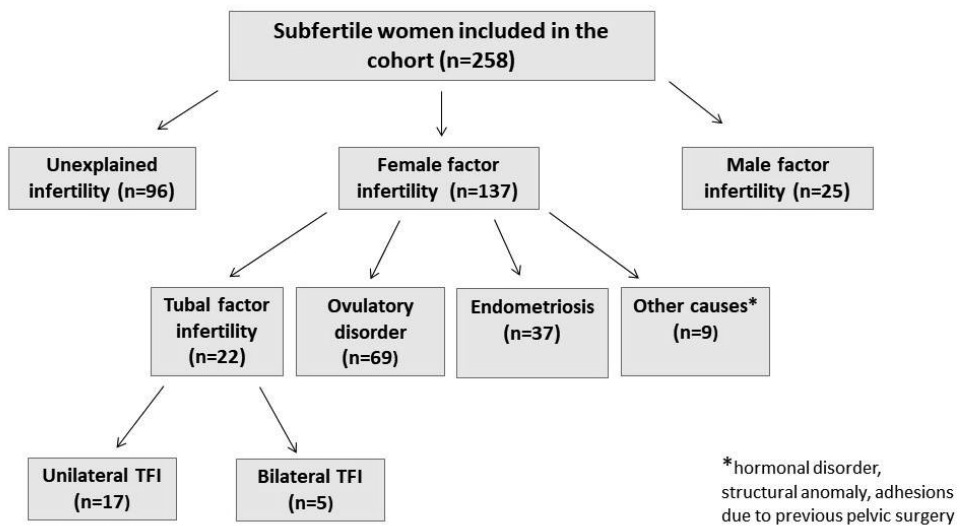
**Figure 7.** Flowchart of the study population in prospective cohort studies. Ten women were excluded for the following reasons: not meeting the criteria for infertility evaluation (n=5), not willing to have infertility evaluation (n=2), being referred directly to IVF from another clinic (n=3).



Altogether, 213 (82.6%) of the 258 women were followed from the first infertility clinic visit to the first delivery or until June 2014 (Study II). The outcomes were clinical pregnancy at 6–7 weeks' gestation, fertility therapy, and the result of the consequent pregnancy. Data on the pregnancy and delivery were collected from the patient registers of Helsinki University Hospital.

TFI was diagnosed in 22 (8.5%) women. The main etiologies for subfertility in the study population after are presented in Figure 8.

**Figure 8.** The main etiologies for subfertility in the study population.



Study IV consisted of 96 women with unexplained infertility. Unexplained infertility was diagnosed in cases where the cause of subfertility remained unknown after the infertility workup. Those women (n=24) who conceived spontaneously before tubal evaluation were also included in this cohort. Altogether, 85 (88.5%) of the 96 women were followed from the first infertility clinic visit to the first delivery or until June 2014. The primary endpoints were clinical pregnancy, time to pregnancy, given fertility therapy, and outcome of the consequent pregnancy.

## 2. Laboratory methods

### 2.1 Serology

In Study I, serum IgG antibodies against *C. trachomatis* major outer membrane protein (MOMP) were measured by a commercially available enzyme immunoassay (EIA) technique (AniLabsystems®, Helsinki, Finland) at the National Institute for Health and Welfare in Oulu, Finland. In Studies II and IV, *C. trachomatis* MOMP-specific and cHSP60-specific serum IgG antibodies were analyzed by commercially available *C. trachomatis* IgG pELISA and cHSP60 IgG ELISA kits (Medac Diagnostika®, Hamburg, Germany). Results were expressed as a mean absorbance at 450 nm. Duplicated samples were analyzed, and a less

than 10% variation was observed in the doublets (OD=optical density >0.2). The cut-off value for a positive antibody result (mean OD of the negative control + 0.350) was OD>0.4.

In Study III, IgG antibody responses to the recombinant *C. trachomatis* TroA and HtrA were analyzed by in-house EIA at the Department of Virology and Immunology, University of Helsinki, Helsinki, Finland (Hokynar *et al.*, 2017). Genes encoding TroA (CT067) and HtrA (CT823) were amplified from *C. trachomatis* serovar D (strain UW-3/Cx ATCC VR-885), cloned, and expressed in *E. coli* as GST-tagged proteins. The expressed proteins were purified by Glutathione Sepharose 4B (GE Healthcare®) according to the manufacturer's instructions for batch purification, and sepharose-bound TroA and HtrA were eluted by glutathione. The protein concentration (A<sub>280</sub>) of each eluate was measured by NanoDrop, and the purity was evaluated by SDS-PAGE and western blotting (Hokynar *et al.*, 2017). Purified TroA and HtrA proteins were used as antigens in EIA, and A<sub>450nm</sub> was measured. Each serum (1:200) was analyzed in duplicate on antigen-coated wells, and the background for each serum was determined by analyzing all sera in duplicate in non-coated wells. The cut-off values were based on the absorbance values (mean +2SD) obtained from specimens of sexually inexperienced girls not exposed to *C. trachomatis* and were set as A<sub>450nm</sub> 0.5.

Sera from the study population were also studied using a microimmunofluorescence test (MIF) (Study III). MIF serology was performed using purified elementary bodies (EB) of *C. trachomatis* and *C. pneumoniae* as the antigen (Gencay *et al.* 2000). MIF titers were classified as high (≥128) or low (≤64).

## 2.2 Cell-mediated immune response

*C. trachomatis*-specific cell-mediated immune response (CMI) for Studies II and IV was analyzed using a lymphocyte proliferation assay at the National Institute for Health and Welfare in Oulu, Finland. Viable cells were available from 234 (90.7%) of the 258 patients. *C. trachomatis* EB (serovars E and F) and recombinant cHSP60 were used as lymphocyte-stimulating antigens (Öhman *et al.*, 2006). Lymphocyte proliferation responses were studied *in vitro* by culture stimulation of peripheral blood mononuclear cells with or without an antigen for 6 days, and tritiated thymidine was added to the cultures for the last 18 h of incubation. Lymphocyte proliferation was measured as counts per minute of incorporated tritiated thymidine in dividing cells and detected by a liquid scintillation counter. The results were obtained as a stimulation index (SI), which was calculated by mean count per minute in the presence of the antigen divided by mean count per minute in its absence for triplicate cultures. SI>5 was considered a positive response to *C. trachomatis* EB, and SI>2.5 was considered a positive response to cHSP60.

### 3. Statistical methods

A chi-squared test was used to compare the seroprevalence rates between the cases and the controls in Study I. Mean absorbance levels of *C. trachomatis* MOMP IgG were compared as continuous variables by using a paired-samples T-test. Due to the skewed distribution of the antibody levels, variables were log-transformed before the analysis. The linear trend in log-transformed antibody levels across the different age-groups was performed by the analysis of variance. To analyze the appropriate measure of the central tendency of skewed distribution, the geometric mean value was calculated by transforming the mean logarithmic values back to the original scale by antilogarithm. Relative risks were assessed by conditional logistic regression, which results in an odds ratio (OR) that estimates the relative risk when the outcome is rare.

For Studies II, III, and IV, a Chi-squared test or Fisher's exact test were used to compare the categorical data, and continuous variables were analyzed with a Student's T-test or Mann-Whitney U-test. To evaluate the performance of *C. trachomatis*-specific immunological markers in TFI prediction (Study II), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), and negative likelihood ratio (LR-) were calculated. An estimated TFI prevalence of 15% was used when post-test probability was analyzed. The performance of immunological tests in TFI prediction was shown as receiver operating characteristic (ROC) curves. For Study III, correlations between *C. trachomatis* and *C. pneumoniae* IgG MIF titers and ranked EIA absorbance values for *C. trachomatis* TroA and HtrA were studied with the Spearman's correlation coefficient. Levels at  $\leq 0.01$  were considered statistically significant. In Study IV, Kaplan-Meier curves were constructed to demonstrate cumulative pregnancy rates according to *C. trachomatis* immunological status.

Statistical analysis for Study I was performed using PASW Statistics 21.0 (SPSS Inc, Chicago, IL) and STATA 5.0 statistical software (Stata Corporation Inc., Stata Statistical Software, College Station, TX). Statistical analyses for Studies II, III, and IV were performed by IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY) and STATA version 13 (Stata Corporation Inc., Stata Statistical Software, College Station, TX). All p-values were two-sided, and  $p < 0.05$  was considered statistically significant.

#### **4. Ethics and permission**

Study I was approved by the ethical committee of the National Institute for Health and Welfare and the FMC steering committee. Permission to obtain information from the health registries was received from the Ministry of Social Affairs and Health (Dnro 86/90/2005).

Studies II, III, and IV were approved by the Helsinki University Hospital Ethical Committee (Dnro 29/E9/07). All couples signed an informed consent before study participation.

# RESULTS

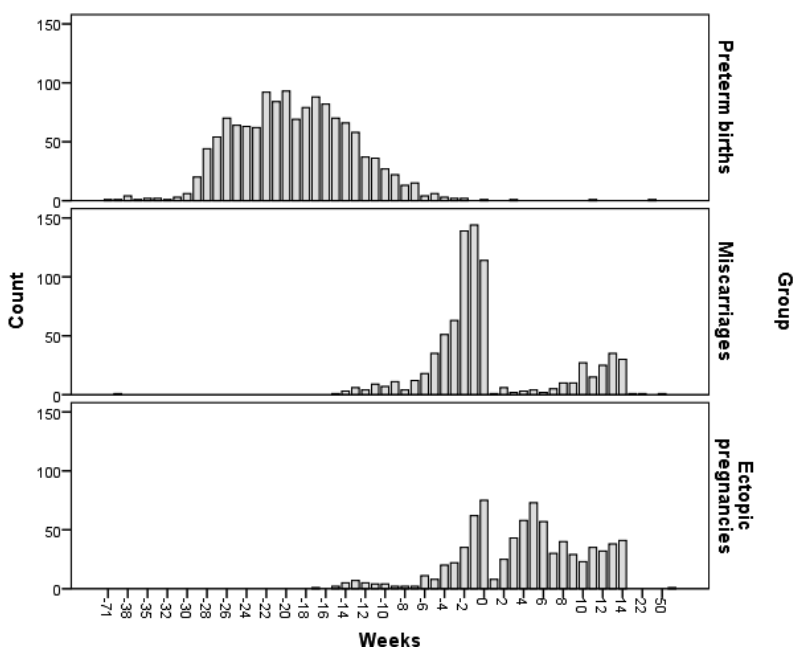
## 1. Population-based register study on *C. trachomatis* and adverse pregnancy outcomes (Study I)

### 1.1. Characteristics of the study population

Altogether, 800 women with ectopic pregnancy (EP), 800 women with miscarriage, 1350 women with preterm delivery (PTD), and an equal number of controls were randomly selected to analyze the serum *C. trachomatis* MOMP antibodies. Most cases with adverse pregnancy outcomes were found in the 20–34 age group (79% EPs, 66% miscarriages, and 76% of PTDs).

Serum samples were obtained from the FMC during the index pregnancy in 38% of cases in the EP group, 79% in the miscarriage group, and 100% in the PTD group, respectively. The time frame of sampling in relation to the occurrence of each clinical outcome is presented in Figure 9.

**Figure 9.** Time between sampling and the clinical endpoint (week 0).

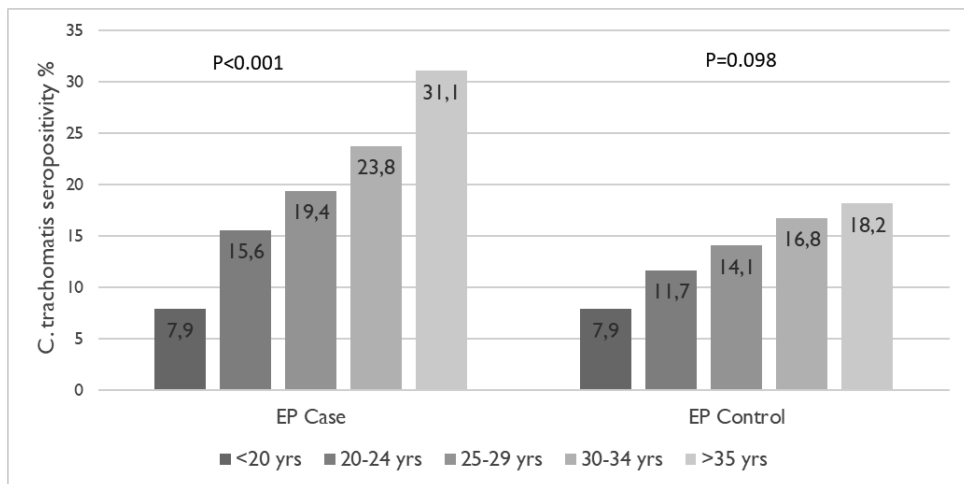




## 1.2. The prevalence of *C. trachomatis* IgG antibodies

The presence of serum antichlamydial IgG antibodies was associated with EP in the index pregnancy samples (29.3% vs. 15.0%,  $p < 0.001$ ; OR 2.30 [95% CI 1.53–3.47]) and in all samples (21.0% vs. 14.6%,  $p = 0.001$ ; OR 1.56 [95% CI 1.2–2.0]). *C. trachomatis* seroprevalence and the serum antibody level increased according to the age of the woman in the EP cases ( $p < 0.001$ ), but not significantly in the controls ( $p = 0.098$ ). The seroprevalence of *C. trachomatis* among the cases and the controls is presented in Figure 10.

**Figure 10.** The prevalence of *C. trachomatis*-specific IgG antibody in the cases with EP and in the controls.

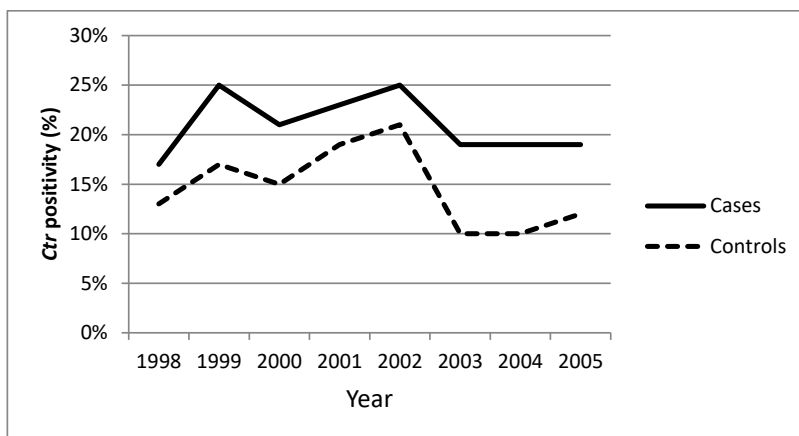


There was no difference in the presence of serum *C. trachomatis* IgG antibodies between the miscarriage and control groups (16.3% vs. 16.8%,  $p = 0.788$ ; OR 0.97 [95% CI 0.74–1.26] in the index pregnancy samples).

The presence of serum *C. trachomatis* antibodies between women with PTD and the control group did not differ (18.1% vs. 18.1%,  $p = 0.960$ ; OR 1.00 [95% CI 0.82–1.21] in the index pregnancy samples). *C. trachomatis* seroprevalence was also analyzed separately in different gestational age groups (<28, 28-31+6, 32-36+6), and no difference was found between cases and controls in any group (results not presented).

*C. trachomatis* seroprevalence in EP cases and the controls were analyzed annually during 1998–2005. Throughout the study period, the seroprevalence was approximately 5–10 % units higher among the cases than in the controls (Figure 11).

**Figure 11.** Annual prevalence of *C. trachomatis*–specific IgG antibodies (%) in the cases with EP and in the controls. CTR=*C. trachomatis*.



## 2. Prospective cohort studies on *C. trachomatis*–specific immune response and subfertility (Studies II, III, and IV)

### 2.1 Characteristics of the study population

Altogether, 37 (14.3%) of 258 women reported a prior history of at least one *C. trachomatis* infection, and 6 (2.3%) reported having two or more infection episodes. Three (1.2%) women reported a history of PID. The rates of having at least one chlamydia episode (18.2% vs. 14.2%  $p=0.54$ ), recurrent episodes (4.5% vs. 2.2%,  $p=0.42$ ), or a history of PID (4.5% vs. 0.8%,  $p=0.24$ ) did not statistically differ between women with TFI and other causes for subfertility. None of the participants had positive *C. trachomatis* NAAT at the time of the infertility workup.

Women with TFI were older (33.1 years vs. 31.2 years,  $p=0.04$ ), had secondary infertility (54.5% vs. 27.1%,  $p=0.007$ ), and were more likely to be smokers (38.1% vs. 16.0%,  $p=0.01$ ) compared to women with other causes for subfertility.

Altogether, 96 (37.2%) of the 258 women were diagnosed with unexplained infertility after their workup. The mean age of women with unexplained infertility was 31.7 (20.7–39.5) years.

## 2.2 The prevalence of *C. trachomatis*-specific immune markers in the study population (Studies II and III)

Serum IgG antibody responses were studied using *C. trachomatis* MOMP, TroA, HtrA, and cHSP60 as antigens. Cell-mediated immune responses were studied *in vitro* by analyzing lymphocyte proliferation responses against *C. trachomatis* EB and cHSP60. Altogether, 94 (36.4%) of the 258 women had serum IgG antibodies against at least one of the chlamydial antigens studied (MOMP, TroA, HtrA, or cHSP60). All serological markers were more frequently positive in the TFI group than in women with other etiologies for subfertility, but the presence of *C. trachomatis*-induced cell-mediated immune markers did not differ between the groups (Table 4). The prevalence of serum antibodies to *C. trachomatis* increased with the increasing severity of tubal damage ( $p=0.002$ ), and all women with bilateral TFI ( $n=5$ ) had serum antibodies against at least one of the *C. trachomatis* antigens.

**Table 4.** Prevalence of *C. trachomatis*-induced humoral and cell-mediated immunological markers in the study population.

	TFI (n=22)	Non-TFI subfertility (n=236)	p-value
<b>Serological markers (n, %)</b>			
<i>C. trachomatis</i> MOMP IgG	9 (40.9)	29 (12.3)	<0.001
cHSP60 IgG	6 (27.3)	24 (10.2)	0.02
<i>C. trachomatis</i> TroA IgG	10 (45.5)	45 (19.1)	0.004
<i>C. trachomatis</i> HtrA IgG	8 (36.4)	31 (13.2)	0.004
<b>Cell-mediated immune markers* (n, %)</b>			
<i>C. trachomatis</i> EB LPR	18 (85.7)	143 (67.1)	0.08
cHSP60 LPR	5 (23.8)	61 (28.6)	0.64
*data on CMI response missing in 24 cases			

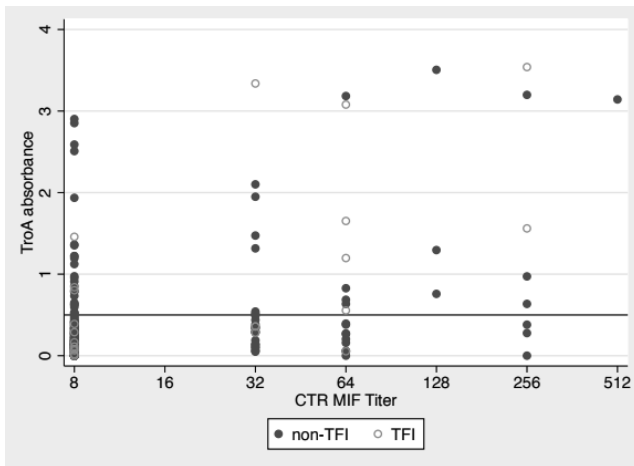
*C. trachomatis* TroA and HtrA EIA antibody levels were compared to *C. trachomatis* IgG antibody MIF titers in Study III. Of the samples with high MIF titers ( $\geq 128$ ), 75.0% were TroA antibody-positive and 58.3% were HtrA antibody-positive. The correlation coefficient for MIF titers and TroA IgG was 0.302 (for TFI patients 0.555 and for non-TFI patients 0.250). For MIF titers and HtrA antibody level, the correlation coefficient was 0.336 (for TFI 0.570 and for non-TFI 0.291) (Figure 12).

To analyze the specificity of *C. trachomatis* TroA and HtrA EIA assays, *C. pneumoniae* IgG titers were determined by MIF and compared to TroA and HtrA. Correlations were weak

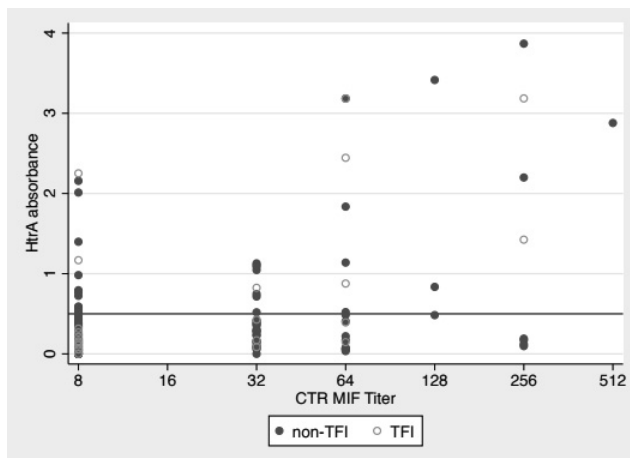
between *C. pneumoniae* antibodies and TroA or HtrA antibodies, which supports the specificity of the assay. The Spearman correlation coefficients for those were 0.053 ( $p=0.40$ ) for TroA and 0.079 ( $p=0.21$ ) for HtrA IgG, respectively.

**Figure 12.** Correlation between *C. trachomatis* TroA IgG (A) and HtrA IgG (B) absorbance values and *C. trachomatis* IgG MIF titers. Women with TFI (open circles) and non-TFI (dark gray circles) were plotted by TroA and HtrA IgG mean  $A_{450nm}$  levels and a *C. trachomatis* IgG MIF titer. The cut-off value for the seropositivity of TroA and HtrA was 0.5, marked with the gray line. In TFI patients, the correlation coefficient was moderate between TroA IgG and MIF titers ( $r=0.555$ ,  $p<0.01$ ) (A) and between HtrA IgG and MIF titers ( $r=0.570$ ,  $p<0.01$ ) (B).

A.



B.



### 2.3 Outcomes of the follow-up (Study II)

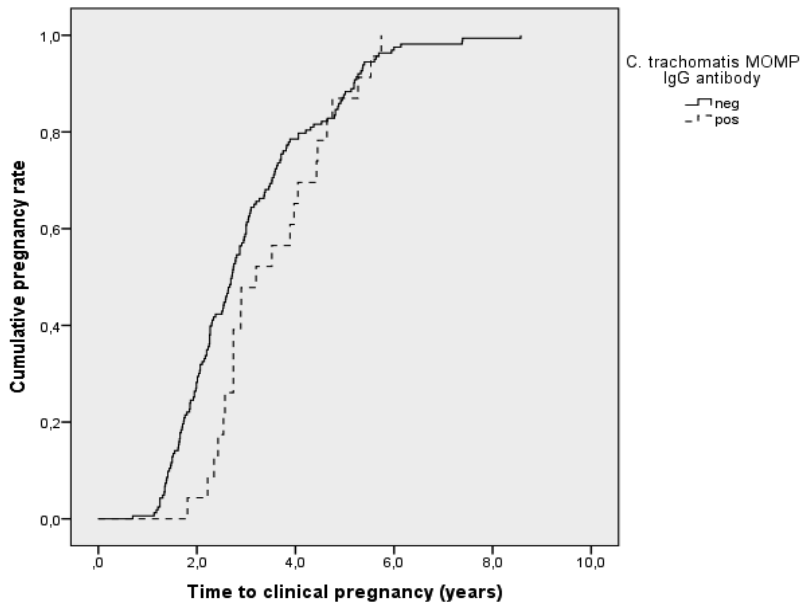
Of the 258 subfertile women, 213 were followed (range 2.1–80.5 months). Altogether, 188 (88.3%) of the 213 women achieved clinical pregnancy and 185 (86.9%) achieved live birth. 71 (38.4%) of the 185 women conceived spontaneously, 55 (29.3%) by OI or OI combined with IUI, and 59 (31.4%) by IVF. Live birth rate (LBR) was significantly ( $p < 0.001$ ) lower in women with TFI ( $n = 10$ , 58.8%) compared to those with another etiology for subfertility ( $n = 175$ , 89.8%). In the TFI group, OI was performed on 5 patients prior to IVF treatment, but none of these women achieved clinical pregnancy following this treatment.

The fertility therapy, clinical pregnancy rate, or LBR according to the presence of *C. trachomatis*-specific humoral or cell-mediated immune response did not differ (Table 6). The time to clinical pregnancy was significantly longer in women with *C. trachomatis* MOMP IgG antibodies than in women without (median 3.2 years [range 1.8–5.7] vs. 2.7 years [range 0.7–8.6],  $p = 0.03$ ) (Figure 13).

**Table 6.** Outcome of the pregnancies by *C. trachomatis*-induced humoral and cell-mediated immune response.

Pregnancy outcomes	<i>C. trachomatis</i> MOMP IgG antibody			<i>C. trachomatis</i> EB LPR*		
	positive (N=38)	negative (N=220)	<i>P</i> -value	positive (N=161)	negative (N=73)	<i>P</i> -value
Live birth rate, n (%)	23 (82.1)	162 (87.6)	0.43	18 (81.8)	151 (88.3)	0.38
Spontaneous pregnancy, n (%)	9 (39.1)	62 (38.3)	0.94	8 (44.4)	57 (37.7)	0.58
Treatment-related pregnancy, n (%)	14 (60.9)	100 (61.7)	0.94	10 (55.6)	94 (62.3)	0.58
Ovulation induction, n (%)	5 (21.7)	50 (30.9)	0.37	5 (27.8)	47 (31.1)	0.77
IVF, n (%)	9 (39.1)	49 (30.2)	0.39	5 (27.8)	47 (31.1)	0.77
*LPR=lymphocyte proliferation, data missing in 16 cases						

**Figure 13.** Cumulative pregnancy rate for *C. trachomatis* IgG seropositive (N=23, dashed line) and seronegative (N=165, black line) women.



#### 2.4 Diagnostic value of *C. trachomatis*-induced immunological markers in TFI prediction (Study II)

The diagnostic value of *C. trachomatis* MOMP IgG, cHSP60 IgG, and cell-mediated immune markers in TFI prediction was studied alone and in combinations (Table 7). The best single test was *C. trachomatis* MOMP, with a specificity of 40.9% and a sensitivity of 87.7%. The combination of *C. trachomatis* MOMP and the cHSP60 IgG antibody had the best positive predictive value for TFI (LR+ of 6.7 and PPV of 38.5%), but the LR- was 0.8, indicating some false negative cases.

**Table 7.** Test performances of *C. trachomatis*–induced serological and cell-mediated immune (CMI) markers in TFI prediction. Post-test probability of TFI was calculated using a 15% TFI prevalence. LPR=lymphocyte proliferation assay

Test	Sensitivity	Specificity	Accuracy	PPV	NPV	LR+	LR-	Positive post-test probability	Negative post-test probability
	%	%	%	%	%			%	%
<b>Serological markers</b>									
MOMP IgG	40.9	87.7	83.7	23.7	94.1	3.3	0.7	37.0	10.6
CHSP60 IgG	27.3	89.9	84.5	20.0	93.0	2.7	0.8	32.1	12.5
<b>CMI markers</b>									
LPR EB	32.7	85.7	37.6	11.2	95.9	0.8	1.1	18.4	7.1
LPR cHSP60	23.8	71.4	67.1	7.6	90.5	1.3	0.4	12.8	15.9
<b>Combinations</b>									
MOMP IgG + CHSP60 IgG	22.7	96.6	90.3	38.5	93.0	6.7	0.8	54.2	12.5
MOMP IgG + LPR EB	38.1	90.1	85.5	27.6	93.7	3.9	0.7	40.5	10.8

## 2.5 The role of *C. trachomatis* in unexplained infertility (Study IV)

Altogether, 96 (37.2%) of the 258 women were diagnosed with unexplained infertility. The mean age of the women was 31.7 (20.7–39.5) years. Of these women, 11 (11.5%) had *C. trachomatis* MOMP IgG antibodies and 57 (59.4%) had a cell-mediated immune response against *C. trachomatis* EB.

Altogether, 85 (88.5%) of the 96 women were followed (Table 8). During the follow-up, 78 (92.0%) of the 85 women had live births. Of those 78, 41 (52.6%) became pregnant spontaneously, 13 (16.7%) by OI or OI combined with IUI, and 24 (30.8%) by IVF. The overall spontaneous or treatment-related pregnancy rate did not differ by the immunological status to *C. trachomatis*. The time to spontaneous pregnancy was longer in women with antichlamydial MOMP antibodies than in those without (median 2.9 years [range 2.4–4.1] vs. 2.0 years [range 1.2–5.3],  $p=0.03$ ). The time to spontaneous pregnancy did not differ between women with or without cell-mediated immune response to *C. trachomatis* (median 2.1 years [range 1.2–5.3] vs. 2.0 years [range 1.3–3.5],  $p=1.00$ ).

**Table 8.** Outcome of pregnancies in women with unexplained infertility

Pregnancy outcomes in women with unexplained infertility	<i>C. trachomatis</i> MOMP IgG			<i>C. trachomatis</i> EB LPR*		
	Positive (N=11)	Negative (n=74)	<i>P</i> -value	Positive (N=52)	Negative (n=26)	<i>P</i> -value
Spontaneous pregnancy, n (%)	5 (55.6)	36 (52.2)	1.00	27 (56.3)	11 (45.8)	0.40
OI+IUI-related pregnancy, n(%)	1 (11.1)	12 (17.4)	1.00	10 (20.8)	3 (12.5)	0.52
IVF-related pregnancy, n (%)	3 (33.3)	21 (30.4)	1.00	11 (22.9)	10 (41.7)	0.10
Time to spontaneous pregnancy (y)						
Mean (range)	3.0 (2.4–4.1)	2.2 (1.2–5.3)	0.03	2.4 (1.2–5.3)	2.1 (1.3–3.5)	0.57
Median	2.9	2.0		2.1	2.0	
Live birth rate, n (%)	9 (81.8)	69 (93.2)	0.22	48 (92.3)	24 (92.3)	1.00

\*LPR=lymphocyte proliferation



## **DISCUSSION**

The increasing incidence of *C. trachomatis* infection is a global concern due to its potentially detrimental effects on reproductive health. The risk of long-term sequelae increases especially with recurrent chlamydial infections and in some women, *C. trachomatis* represents a major threat to fertility. The aim of this thesis was to elucidate the impact of *C. trachomatis* infection on adverse pregnancy outcomes and subfertility by analyzing immune responses against *C. trachomatis*. Another aim was to develop a non-invasive test to detect tubal factor infertility (TFI) to simplify the infertility workup.

According to our results, past *C. trachomatis* infection is associated with ectopic pregnancy (EP) but may play a less significant role in the etiology of EP than previously assumed. We found no serological link between *C. trachomatis* infection and miscarriage or preterm delivery (PTD). Serum IgG antibodies to *C. trachomatis* were more prevalent among women with TFI than in subfertile women with other causes for subfertility, but in TFI screening, *C. trachomatis*-specific immune markers were only of modest value. Past *C. trachomatis* infection did not have an impact on pregnancy outcomes in women with unexplained infertility, but the time to spontaneous pregnancy was longer among *C. trachomatis* IgG seropositive women than in seronegative women.

### **1. Population-based register study on *C. trachomatis* and adverse pregnancy outcomes (Study I)**

A number of case-control studies have demonstrated a strong link between past *C. trachomatis* infection and EP (Brunham *et al.*, 1992a, Chow *et al.*, 1990, Sziller *et al.*, 1998), but only a few studies have evaluated the risk of EP following chlamydial infection at the population level. Although the link between *C. trachomatis* infection and pelvic inflammatory disease (PID) has been well established (Low *et al.*, 2006, Oakeshott *et al.*, 2010), linking chlamydial infection to EP is not straightforward. Uniform diagnostic criteria for PID are lacking, and even after ascending to the upper genital tract, *C. trachomatis* infection is usually cleared without reproductive sequelae (Gottlieb *et al.*, 2010). Previous landmark studies on which the risk of *C. trachomatis*-associated EP have been mainly based have suffered from several bias problems (Barlow *et al.*, 2001, Brunham *et al.*, 1992a, Sziller *et al.*, 1998). Firstly, they have been mostly clinical case-control studies performed on small study populations. Furthermore, they have included women with severe chlamydial infections, which are more likely to result in long-term morbidity.

Our study, along with other population-based studies, suggests that the long-term risks following chlamydial infection are lower than previously assumed (Bender *et al.*, 2011, Davies *et al.*, 2016, Low *et al.*, 2006). In a recent population-based study from Denmark,

the lifetime risk of EP in women with at least one diagnosed *C. trachomatis* infection was 2.2%, compared to 2.0% of those who tested negative and to 0.6% of those never tested (Davies *et al.*, 2016). In another study that evaluated the cost-effectiveness of *C. trachomatis* screening, the estimated probability of EP in women with current chlamydial infection was 0.07% (van Valkengoed *et al.*, 2004).

The serological link between *C. trachomatis* infection and EP was observed in our study. The association between serum *C. trachomatis* IgG antibodies and EP increased with age and was strongest in women over 35 years old. The prevalence of serum *C. trachomatis* IgG antibodies and antibody levels were highest in older women, reflecting a history of repeat exposures to *C. trachomatis*, which is a known risk factor for EP (Haggerty *et al.*, 2010, Hillis *et al.*, 1997). However, only one-fifth of the EP cases had antichlamydial IgG antibodies, suggesting that past chlamydial infection may play a less significant role in the etiology of EP than previously thought.

The overall rate of EP has declined in Finland and other high-income countries since the 1990s (Moore *et al.*, 2016, National Institute for Health and Welfare, 2016), which may result from successful *C. trachomatis* screening programs and the reduction in the incidence of PID (Rekart *et al.*, 2013). Also, factors other than the effective *C. trachomatis* screening may have driven the declining trend in EP rates. More careful sexual behavior as a response to the global HIV epidemic and STI prevention campaigns may have restricted the transmission of PID-related pathogens. Importantly, due to voluntary childlessness, the birth rate in Finland has been constantly declining since the 1990s, leading to the declining trend of all adverse pregnancy outcomes reported to national registers.

*C. trachomatis* persistence in the upper genital tract has been linked to increased risk for miscarriage or PTD (McGregor and French, 1991). In our study, no serological association was found between *C. trachomatis* infection and miscarriage. It has been hypothesized that *C. trachomatis* may induce miscarriage through immunological mechanisms or endometrial damage, but these studies have been performed with limited study populations (Quinn *et al.*, 1987, Witkin and Ledger, 1992). Serological evidence between *C. trachomatis* infection and miscarriage has been suggested (Wilkowska-Trojnieł *et al.*, 2009), but most studies have not found such a link (Paukku *et al.*, 1999, Rae *et al.*, 1994, Sugiura-Ogasawara *et al.*, 2005). *C. trachomatis* DNA has been found more frequently in products of conception from women with miscarriage than in controls, suggesting that acute *C. trachomatis* infection rather than past infection could result in early pregnancy loss (Baud *et al.*, 2011). In general, infections account for up to 15% of early miscarriages (Giakoumelou *et al.*, 2016). Since the association between *C. trachomatis* and other pathogens colonizing vaginal flora has been well established (Tamarelle *et al.*, 2018, Wiesenfeld *et al.*, 2003), serum antichlamydial antibodies may actually reflect the presence of other potentially virulent organisms in the upper reproductive tract and are capable of causing adverse pregnancy outcomes.

*C. trachomatis* infection during pregnancy has been connected to PTD in several studies (Andrews *et al.*, 2000, Goldenberg *et al.*, 2000). The serological link has also been observed: serum IgM antibodies against *C. trachomatis* have been linked to PTD, but IgG antibodies have not (Gencay *et al.*, 2000). In a population-based study from Finland, the simultaneous presence of *C. trachomatis* IgG antibodies and elevated hsCRP during pregnancy increased the risk for PTD 4.3-fold (95% CI 2.0–9.3) (Karinen *et al.*, 2005). These results suggest that acute chlamydial infection during pregnancy or chronic infection with inflammatory response may play a role. We found no association between PTD and serum *C. trachomatis* IgG antibodies, but we did not study inflammatory markers. The presence of antichlamydial IgG antibodies in our study may reflect past exposure to *C. trachomatis* rather than present infection or inflammation that could induce PTD. Thus, although we could not find a serological link between *C. trachomatis* and PTD, we cannot rule out acute chlamydial infection during pregnancy as a cause of preterm birth.

## **2. Using immune markers for *C. trachomatis* to predict TFI (Studies II and III)**

Serum antichlamydial IgG antibodies have been linked to TFI in several studies (Cates and Wasserheit, 1991, Freidank *et al.*, 1995, Punnonen *et al.*, 1979). In our study, humoral immune response to *C. trachomatis* was associated with TFI, but cell-mediated immune response was not.

Tubal tissue damage is suggested to be developed by cell-mediated immunological mechanisms (Brunham *et al.*, 1992a, Kinnunen *et al.*, 2002, Öhman *et al.*, 2006). In our study, *C. trachomatis*-specific cell-mediated immune response was also positive in many subfertile women without tubal damage. Uncomplicated, single chlamydial infection with early treatment may elicit a cell-mediated immune response, but it is unlikely that tubal damage has occurred in these patients. Furthermore, not every individual exposed to *C. trachomatis* develops a clinical infection (Molano *et al.*, 2005, Morr e *et al.*, 2002).

Immune response to cHSP60 has been strongly linked to chronic chlamydial infection and TFI (Claman *et al.*, 1997, den Hartog *et al.*, 2005, Toye *et al.*, 1993). In our study, the prevalence of cHSP60 IgG antibodies and cHSP60-induced cell-mediated immune response was surprisingly low, suggesting that cHSP60 is a poorer marker of *C. trachomatis*-related TFI than previously assumed.

Accurate, non-invasive methods for TFI prediction in early infertility workup would be clinically valuable in identifying cases with TFI to avoid unnecessary investigations and delay in treatment, or in planning the most effective and economical treatment for a subfertile couple. According to our results, cHSP60 IgG antibody had the highest specificity in predicting TFI, but the test sensitivity was low because of many cHSP60 IgG negative cases. *C. trachomatis* MOMP IgG test had the best sensitivity for predicting TFI, but it had

a relatively low specificity, suggesting many false-positive cases among women with non-TFI subfertility. We found that the specificity of *C. trachomatis* MOMP IgG can be improved by combining *C. trachomatis* MOMP IgG and cHSP60 IgG antibody or by combining *C. trachomatis*-specific cell-mediated immune response with MOMP IgG and cHSP60 IgG antibody tests. According to our results, these combinations would be moderately useful markers to rule in women with TFI in clinical practice. However, they would not be suitable to rule out women with TFI because some of the TFI cases remained as false negatives. These women probably had TFI caused by other factors, or they had not developed a detectable immunological response to *C. trachomatis*. Using immunological markers in TFI prediction in clinical practice is not easy, because the host response to *C. trachomatis* infection varies considerably (Witkin *et al.*, 2000) and the negative antibody titers are not a definitive marker of a negative history of *C. trachomatis* infection (Horner *et al.*, 2013).

The prevalence of *C. trachomatis*-specific immune markers has been compared between TFI cases and healthy controls in previous studies (Öhman *et al.*, 2009, Tiitinen *et al.*, 2006). In our study, we compared TFI cases to other subfertile women, because we wanted to develop a test suitable for clinical practice to detect women with TFI among unselected subfertile women. *C. trachomatis* IgG antibodies are also common in subfertile women with patent tubes (Coppus *et al.*, 2011), and it can be assumed that the difference in the presence of *C. trachomatis*-specific antibodies would have been higher, if we had compared TFI cases to fertile controls. Furthermore, previous studies (Öhman *et al.*, 2009, Tiitinen *et al.*, 2006) included TFI cases diagnosed only by laparoscopy and with more severe forms of TFI (i.e. hydrosalpinges).

In recent years, persistent *C. trachomatis* infection has been the focus of chlamydia research, because persistence is considered one of the key factors in the process leading to tubal damage (Witkin *et al.*, 2017). This immunological process is likely to be caused by certain *C. trachomatis* antigens and other immune mediators (Menon *et al.*, 2015). However, the course of a single *C. trachomatis* infection varies among individuals. While some individuals are more susceptible to the ascent and persistence of *C. trachomatis* infection in the upper genital tract, most women clear the infection without any reproductive consequences. The individual factors influencing the tissue damage and development of TFI are strongly related to the host immunogenetic factors (Jansen *et al.*, 2016, Öhman *et al.*, 2006). Also, environmental factors such as co-microbes colonizing cervicovaginal flora influence the clinical course of chlamydial infection and the development of TFI (Aiyar *et al.*, 2014, Ziklo *et al.*, 2016).

*C. trachomatis* TroA and HtrA are proteins suggested to be essential for the survival of *C. trachomatis* under stressful conditions and are expressed during persistent infection (Wyrick, 2010). It has been shown that IgG antibody response against TroA and HtrA is associated with ascending (Hokynar *et al.*, 2017) or repeat (Stansfield *et al.*, 2013) chlamydial infection rather than with uncomplicated, single infection. TroA and HtrA serology has been suggested as a potential biomarker for reproductive sequelae of *C.*

*trachomatis* infection (Stansfield *et al.*, 2013). We observed that serum IgG antibodies to both TroA and HtrA were more common in women with TFI than in women with other causes for subfertility. Furthermore, the seropositivity rates and serum antibody levels increased with the increasing severity of tubal damage. As a result, we suggest that these biomarkers could be further developed into a more accurate test for TFI prediction than traditionally used CAT serology.

The majority of TFI cases are linked to prior PID, but most women with TFI and antichlamydial IgG antibodies do not have a history of clinical PID. This suggests that many subclinical or mild PID cases may go undetected (Wiesenfeld *et al.*, 2012). Data from prospective studies indicate that about 10–15% of untreated genital *C. trachomatis* infections will progress to PID in one year (Haggerty *et al.*, 2010, Oakeshott *et al.*, 2010, Price *et al.*, 2016). The *C. trachomatis*-associated proportion of TFI has been estimated at around 29–45% and repeat episodes of chlamydial infections significantly increase the risk of tubal damage (Ades *et al.*, 2017, Weström *et al.*, 1992).

Despite the robust evidence of chlamydia association with TFI, not all women with TFI are infected with *C. trachomatis*. Tubal pathology resulting from PID may be caused by other sources of infection, including *N. gonorrhoeae* (Morales *et al.*, 2006), *Mycoplasma genitalium* (Wiesenfeld and Manhart, 2017), and BV-associated anaerobic bacteria (Haggerty *et al.*, 2016). Previously, most PID cases were thought to be caused by *C. trachomatis* (Mårdh *et al.*, 1977, Paavonen, 1980), but according to current opinion, most women with PID are not infected with chlamydia (Brunham *et al.*, 2015). In recent studies, *C. trachomatis* was detected in only 15–30% of PID cases (Goller *et al.*, 2016). The multifactorial etiology of TFI is likely to result in low a sensitivity of *C. trachomatis* antibody tests in TFI screening and is often ignored when evaluating the performance of *C. trachomatis* serology in TFI prediction.

Tubal damage can vary from visible intra-tubal adhesions and hydrosalpinges to milder forms without visible tubal occlusion (Akande, 2007). Laparoscopy has been considered a gold standard in TFI evaluation, but it is invasive, costly, and can cause complications. In our study, tubal evaluation was mainly performed by HSSG, which is known to have limited accuracy and low inter-observer reproducibility (Mol *et al.*, 2001). Furthermore, peritubal adhesions and impaired tubal function cannot be revealed by this procedure. However, HSSG has a high sensitivity for intrauterine pathologies or abnormalities in ovaries, which is important in relation to subfertility (Maheux-Lacroix, 2014). It has been suggested that diagnostic tubal patency testing may also improve fertility by tubal flushing (Dreyer *et al.*, 2017, Luttjeboer *et al.*, 2007). In our study, LBR was significantly lower in TFI patients than in those with other causes of subfertility, which is in line with previous studies (Kawwass *et al.*, 2013, Keltz *et al.*, 2013). It has been shown that women with untreated hydrosalpinges are at higher risk of adverse pregnancy outcomes, leading to lower LBR (Camus *et al.*, 1999, Strandell *et al.*, 1999). Lower LBR may also be associated with chronic

endometrial inflammation, resulting in impaired implantation (Bouet *et al.*, 2016, Johnston-MacAnanny *et al.*, 2010).

### 3. The role of *C. trachomatis* infection in unexplained infertility (Study IV)

Studies have suggested that *C. trachomatis* may impair the chance for spontaneous pregnancy, even in the absence of visible tubal occlusion (Coppus *et al.*, 2011, Steiner *et al.*, 2015). An inflammatory response in the upper genital tract may damage Fallopian tube tissue by reducing the number of ciliated mucosal cells and leading to functional tubal damage (Shao *et al.*, 2012). Such dysfunction may result in impaired ovum transport, lower chance for spontaneous pregnancy, and risk of EP. It is also possible that *C. trachomatis* impairs fertility by mechanisms other than damaging Fallopian tubes, such as causing chronic endometritis or post-infectious inflammation in the endometrium (Bouet *et al.*, 2016).

To investigate the role of *C. trachomatis* in unexplained infertility, we studied both humoral and cell-mediated immune responses against *C. trachomatis*. We found that neither humoral nor cell-mediated immune response against *C. trachomatis* were associated with unexplained infertility. The presence of serum antichlamydial IgG antibodies or cell-mediated immune response to *C. trachomatis* had no impact on overall pregnancy rate or LBR in women with unexplained infertility. However, the time to conceive spontaneously was longer in women with antichlamydial IgG antibodies than in women without. Serum antichlamydial antibodies had no impact on the outcomes of treatment-related pregnancies, which agrees with previous studies that showed no differences in pregnancy rates after IVF treatment between *C. trachomatis* IgG negative or positive women (de Barbeyrac *et al.*, 2006, Spandorfer *et al.*, 1999).

Our result of a prolonged time to spontaneous pregnancy in *C. trachomatis* seropositive women agrees with a large multicenter study from The Netherlands (Coppus *et al.*, 2011), which found that women with unexplained infertility and serum antichlamydial antibodies had lower spontaneous pregnancy rates over 12 months than seronegative women. El Hakim *et al.* found no association between *C. trachomatis* seropositivity and pregnancy rates at the three-year follow-up in women with unexplained infertility, but they did not study the time to spontaneous conception (El Hakim *et al.*, 2009).

Our study is the first to investigate the role of a *C. trachomatis*-induced cell-mediated immune response in unexplained infertility. Contrary to women with serum antichlamydial IgG antibodies, women with *C. trachomatis*-induced cell-mediated immune response did not have a prolonged time to natural conception. This finding agrees with Study II, where the best immunological marker in identifying women with TFI was a humoral response to *C. trachomatis*.

#### 4. Strengths and limitations

The major strength of our register-based biobank study was the population-based data with a large number of patients. Finland has excellent health registries and the FMC serum bank, which covers almost all pregnant women in Finland. However, FMC lacks infertile women and adolescents who have not yet been pregnant. In addition, serum samples in the FMC are collected during the first antenatal clinic visit, and many miscarriages or ectopic pregnancies occur earlier. We could not evaluate the possible confounding factors in our study, such as sexual and lifestyle behaviors, socioeconomic factors, infertility treatment, and other pathogens causing PID and subsequent reproductive problems.

The overall rate of serum antichlamydial antibodies was surprisingly low in our studies. The seroprevalence of *C. trachomatis* in the population has declined over time (Lyytikäinen *et al.*, 2008b), suggesting that the majority of reported *C. trachomatis* infections are uncomplicated infections leading to a mild humoral immune response and low antibody production (Brunham *et al.*, 2005, Ngeow, 1996). Also, the persistence of detectable antibody titers in serum is somehow unclear. It has been suggested that antibody levels may fade over time after uncomplicated *C. trachomatis* infection, especially if the antibody titers were originally low (Horner *et al.*, 2013).

TFI prevalence was low in our study (8.5%), which led to small study groups. This is in line with the overall declining TFI trend in the population (Kawwass *et al.*, 2013, National Institute for Health and Welfare, 2016). In high-income countries, TFI is estimated to account for 10–30% of the cases of subfertility (Healy *et al.*, 1994, Tsevat *et al.*, 2017, Wiesenfeld *et al.*, 2012), but due to the variability in TFI definition and divergent methods in TFI diagnostics, reliable national TFI surveillances are not achievable in many countries. The declined TFI prevalence may be a consequence of successful *C. trachomatis* screening programs, which have led to the effective treatment of chlamydial infections before they ascend to the upper genital tract. It is also possible that the immunobiology of chlamydial infection may have changed so that less virulent *C. trachomatis* strains have spread and become common in the population (Byrne, 2010, Morrison, 2000).

Another limitation of our study was the low accuracy of the HSSG, which was mainly used as a diagnostic method for tubal evaluation. HSSG is known to have limited inter-observer reproducibility, and false positive results are common due to the tubal spasm. Furthermore, peritubal adhesions and impaired tubal function cannot be revealed by this procedure. However, HSSG has great sensitivity in detecting pathologies of the uterine cavity and allows concomitant visualization of the ovaries (Saunders *et al.*, 2011, Maheux-Lacroix *et al.* 2014).

The strength of our study was the solid collaboration with the laboratories of the National Institute of Health and Welfare and the Department of Virology and Immunology, Helsinki University. The in-house EIA method for TroA and HtrA serology and the lymphocyte

proliferation *in vitro* assay are unique methods for studying the impact of the *C. trachomatis*-induced immune response to fertility. Our study was the first to evaluate the combination of humoral and cell-mediated immune responses in predicting TFI in clinical settings. Additionally, we were the first to investigate the impact of the cell-mediated immune response to *C. trachomatis* on pregnancy outcomes. Although our cohort of subfertile women was collected during 2007 and 2010, the diagnostic procedures and management protocols have not considerably changed, which makes our results applicable to the present day.

## 5. Future prospects

Studies estimating the risk of long-term sequelae of genital *C. trachomatis* infection are important, since the worldwide chlamydia epidemic with over 130 million new cases annually (World Health Organization, 2017), does not seem to wane. Future studies should focus more on investigating the individual factors that affect the ascent and persistence of *C. trachomatis* infection in the upper genital tract. Implications using immunogenetic markers as indicators for enhanced immunological response in complicated chlamydial infection would be promising in predicting reproductive sequelae (Jansen *et al.*, 2016, Öhman *et al.*, 2006). Furthermore, as specific features of the vaginal microbiome may indicate an increased risk of acquiring STIs, PID, and potential reproductive sequelae, studies investigating the factors affecting the composition of reproductive tract microbiome are relevant. Since the clinical diagnostics of PID are imprecise, further studies are needed to develop accurate, non-invasive tools to detect upper genital tract inflammation.

The detailed diagnosis of tubal morphology and function in the infertility workup can be questioned. Enormous changes in the infertility treatment protocols over previous decades have improved the worldwide availability of IVF. Thus, the diagnostic question should be changed to a prognostic one. Rather than focus on the detailed detection of morphologic pathologies of the Fallopian tubes, infertility workup should provide a comprehensive fertility evaluation and prognosis of a subfertile couple. Pelvic infections are one of the most important preventable causes of female infertility, and national preventive strategies should be planned to recognize the core group at the highest risk for PID and subsequent TFI. In addition, better tools to identify women with post-infectious infertility and poorer prognosis for spontaneous pregnancy are needed.



## **CONCLUSIONS**

1. Our results confirm the link between past *C. trachomatis* infection and ectopic pregnancy (EP) at the population level. We found no serological association between *C. trachomatis* infection and miscarriage or preterm delivery (PTD). The low prevalence of serum antichlamydial antibodies in women with EP suggests that chlamydial infection may play a less significant role in the etiology of EP than previously assumed.
2. The predictive value of *C. trachomatis* serology in evaluating tubal factor infertility (TFI) among subfertile women can be improved by combining serum *C. trachomatis* MOMP and cHSP60 IgG antibody tests or by combining cell-mediated immune markers with serology. *C. trachomatis* immune markers in the prediction of TFI are only of modest value due to the multifactorial nature of TFI development.
3. Serum antibodies to *C. trachomatis* TroA and HtrA are more common in women with TFI than in subfertile women with another cause for subfertility. *C. trachomatis* TroA and HtrA serology has the potential to be developed as a specific biomarker in predicting *C. trachomatis*-related tubal pathology.
4. Past *C. trachomatis* infection does not play a significant role in unexplained infertility. A positive cell-mediated immune response against *C. trachomatis* is common in women with unexplained infertility, but it does not have an impact on pregnancy outcomes. Time to spontaneous pregnancy in women with unexplained infertility is longer among *C. trachomatis* IgG seropositive women than in seronegative women.

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A handwritten signature in black ink, appearing to read 'Teemu', written in a cursive style.

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## **ORIGINAL PUBLICATIONS**