Chlamydia trachomatis as a risk factor for infertility in women and men, and ovarian tumor development

Annika Idahl
2009
To my family
with love
“Research is to see what everybody else has seen, and to think what nobody else has thought”

Albert Szent-Gyorgyi
1937 Nobel laureate, Medicine
Abstract

Background: *Chlamydia trachomatis* in women is a risk factor for tubal factor infertility and extra uterine pregnancies, but the impact of a *C. trachomatis* infection on male fertility is unclear. It is also hypothesized that persistent infection with *C. trachomatis*, or other microorganisms, might initiate/promote ovarian tumor development. The aims of the thesis were to study whether *C. trachomatis* serum antibodies in women and men had an impact on infertility diagnoses, semen characteristics, pregnancy rates and pregnancy outcomes; furthermore, to explore associations of *C. trachomatis*, and *Mycoplasma genitalium*, plasma antibodies with epithelial ovarian cancer and borderline ovarian tumors, as well as the presence of *C. trachomatis* bacteria, and other microorganisms, in ovarian tissues.

Materials and methods: Papers I and II: 244/226 infertile couples were tested for serum *C. trachomatis* IgG, IgA, IgM and chlamydial Heat Shock Protein 60 (cHSP60) IgG antibodies. *C. trachomatis* IgG positive couples were also tested for *C. trachomatis* DNA in a urine sample. The follow-up period was 14-54 months. 244 spontaneously pregnant women were also tested for serum *C. trachomatis* IgG antibodies. Papers III and IV: Plasma samples from 291 women with epithelial ovarian cancer, borderline ovarian tumors and benign conditions, and plasma samples from 271 healthy controls, were analyzed for *C. trachomatis* IgG, IgA and cHSP60-1 IgG and *M. genitalium* IgG antibodies. Ovarian tissues from 186 women with benign ovaries, borderline ovarian tumors and epithelial ovarian cancer, as well as tissues from the contra lateral ovary in 126 women, were analyzed for the presence of *C. trachomatis*, *M. genitalium*, *Neisseria gonorrhoeae*, HPV and the polyoma viruses BKV and JCV with nucleic acid amplification tests.

Results: Papers I and II: The prevalence of *C. trachomatis* IgG antibodies was higher among infertile than fertile women, and there were 9 couples with ongoing *C. trachomatis* infections. In men, *C. trachomatis* IgG and IgA antibodies were associated with a reduced likelihood to achieve pregnancy for the couple, as well as lower sperm concentration, reduced sperm motility and vitality, increased teratozoospermia index and the occurrence of leukocytes. *C. trachomatis* IgG and cHSP60 IgG antibodies in infertile women were associated with tubal factor infertility, but not with reduced pregnancy rates or outcomes. Paper III: cHSP60-1 IgG antibodies were associated with ovarian cancer belonging to the postulated type II pathogenetic pathway, when plasma samples obtained more than one year prior to diagnosis were analyzed. *M. genitalium* IgG antibodies were associated with borderline ovarian tumors; however a statistical type 1 error cannot be excluded. Paper IV: None of the microorganisms studied were found in the ovarian tissue samples.

Conclusions: *C. trachomatis* IgG and IgA antibodies in the man substantially decreases the chances of the infertile couple to achieve pregnancy, and are associated with subtle negative changes in semen characteristics. *C. trachomatis* IgG and cHSP60 IgG antibodies in the woman are risk factors for tubal factor infertility. Prospective plasma cHSP60-1 IgG antibodies are associated with type II ovarian carcinomas, but *C. trachomatis* bacteria, or the other microorganisms studied, could not be detected in benign, borderline or malignant ovarian tissues.

Keywords: Antibodies; borderline tumors; *Chlamydia trachomatis*; cHSP60; DNA; infertility; ovarian cancer; pregnancy rate; RNA; semen characteristics.
Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


**Abbreviations**

AIH  artificial insemination, husband sperm  
AID  artificial insemination, donor sperm  
ART  assisted reproductive technique  
ASA  antisperm antibodies  
BMI  body mass index  
BOT  borderline ovarian tumor  
cHSP60  chlamydial Heat Shock Protein 60  
*C. pneumoniae*  *Chlamydia pneumoniae*  
*C. trachomatis*  *Chlamydia trachomatis*  
DNA  deoxyribonucleic acid  
EB  elementary body  
EIA  enzyme immuno assay  
ELISA  enzyme linked immuno sorbent assay  
EOC  epithelial ovarian cancer  
FSH  follicle stimulating hormone  
HPV  human papilloma virus  
*H. pylori*  *Helicobacter pylori*  
HRT  hormone replacement therapy  
HSS  hystero salpingo sonography  
Ig  immunoglobulin  
ICSI  intracytoplasmic sperm injection  
IVF  in vitro fertilization  
LAMP-EIA  Lipid associated membrane protein – Enzyme immuno assay  
LGV  lymphogranuloma venerum  
*M. genitalium*  *Mycoplasma genitalium*  
MgRT-PCR  *Mycoplasma genitalium* Real time - PCR  
MIF  microimmunofluorescence  
MOMP  major outer membrane protein  
NAAT  nucleic acid amplification test  
NAFA/ESHRE  Nordic Association for Andrology/European Society of Human Reproduction and Embryology  
*N. gonorrhoeae*  *Neisseria gonorrhoeae*  
NPV  negative predictive value  
NSHDC  Northern Sweden Health and Disease Cohort  
OD  optical density  
OR  odds ratio  
PCR  polymerase chain reaction  
PID  pelvic inflammatory disease  
PPV  positive predictive value  
RB  reticulate body  
RNA  ribonucleic acid  
RR  relative risk, risk ratio  
SSPC  serous surface papillary carcinoma  
STD  sexually transmitted disease
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>STI</td>
<td>sexually transmitted infection</td>
</tr>
<tr>
<td>TFI</td>
<td>tubal factor infertility</td>
</tr>
<tr>
<td>TIC</td>
<td>tubal intraepithelial carcinoma</td>
</tr>
<tr>
<td>TMA</td>
<td>transcription mediated amplification</td>
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<td>TZI</td>
<td>teratozoospermia index</td>
</tr>
</tbody>
</table>
# Table of contents

## Abstract

## Original Papers

## Abbreviations

## Table of contents

### Introduction

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

*Chlamydia trachomatis* – the bacterium ........................................... 2

Developmental cycle ........................................................................ 3

Persistence ......................................................................................... 4

Chlamydial Heat Shock Proteins ........................................................... 4

Clinical manifestations of genital *C. trachomatis* ................................ 5

*C. trachomatis* diagnostic methods ..................................................... 5

  - Test specimen ........................................................................... 5
  - Culture ....................................................................................... 6

  - Immunofluorescence (IF) and antigen detection enzyme immunoassays (EIA) ................................................................. 6
  - Nucleic acid amplification assays – NAATs ........................................ 6

  - Serology ....................................................................................... 7

Effect of antibiotic treatment ............................................................... 8

### Subfertility/infertility

- Definition and prevalence ............................................................. 9
- Psychosocial aspects ...................................................................... 9

**Chlamydia trachomatis** and infertility ............................................... 9

*C. trachomatis* and female infertility ................................................ 9

*C. trachomatis* and male infertility .................................................. 10

### Ovarian tumors

- Prevalence and risk factors .......................................................... 10
- Ovarian tumor biology ................................................................. 11

Pathogenesis of serous ovarian and pelvic carcinomas – a new model ................................................................. 12

### Chronic infection and inflammation in the pathogenesis of ovarian tumors

- Other types of cancer ................................................................. 12

### Aims

- Previous studies .......................................................................... 13

### Other genital infections as possible tumor promoters/initiators

- Fertile controls ............................................................................ 19

## Materials and Methods

- Ethics ............................................................................................ 17

**Papers I and II: Infertility study** .................................................... 17

- Study population ......................................................................... 17

- Clinical investigation .................................................................. 17

- Follow-up ..................................................................................... 19

- Fertile controls ............................................................................ 19
Papers III and IV: Ovarian tumor study ................................................................. 19
Study population ..................................................................................................... 19
Clinical characteristics and histopathologic diagnosis ........................................... 19

C. trachomatis analyses .......................................................................................... 20
C. trachomatis serology .......................................................................................... 20
DNA and RNA extraction ....................................................................................... 21
C. trachomatis DNA and RNA analyses ............................................................... 21

Additional analyses ............................................................................................... 21
Paper III: M. genitalium serology ........................................................................... 21
Paper IV: N. gonorrhoeae, M. genitalium, HPV and polyoma virus BKV and JCV
detection .................................................................................................................. 21

Statistics ................................................................................................................ 22

Results and Discussion ........................................................................................ 23

Papers I and II: Infertility study .............................................................................. 23
Prevalence of C. trachomatis antibodies and DNA .................................................. 23
C. trachomatis antibodies and female infertility ...................................................... 25
C. trachomatis antibodies and male infertility ......................................................... 26
Methodological considerations .............................................................................. 29
Different antibodies – different mechanisms? ......................................................... 30

Papers III and IV: Ovarian tumor study ................................................................. 30
Prevalence of C. trachomatis and M. genitalium antibodies ..................................... 30
C. trachomatis and M. genitalium antibodies in relation to ovarian tumors ............ 32
C. trachomatis, N. gonorrhoeae, M. genitalium, HPV, and polyoma virus BKV and
JCV in ovarian tissues (paper IV) ........................................................................ 33
Methodological considerations .............................................................................. 33
Suggested model of C. trachomatis in the pathogenesis of ovarian cancer .............. 35
  When does C. trachomatis initiate/promote ovarian carcinogenesis? ................. 35
  Where is the site for ovarian carcinogenic C. trachomatis infections? ............... 36
  How does the C. trachomatis infection initiate/promote malignant transformation? 36

Summary and Conclusions .................................................................................... 39

Suggestions for future research ............................................................................. 41

Acknowledgements ............................................................................................... 43

References ............................................................................................................. 45

Papers I–IV
Introduction

*Chlamydia trachomatis* (*C. trachomatis*), the most prevalent sexually transmitted bacteria worldwide, is a known risk factor for pelvic inflammatory disease (PID), tubal factor infertility (TFI) and ectopic pregnancies, but the impact of a *C. trachomatis* infection on male fertility is unclear. It is also recently hypothesized that chronic infections with for example *C. trachomatis* might initiate/initiate/promote ovarian tumor development.

In this thesis known consequences of *C. trachomatis* on female fertility in terms of TFI will be confirmed. Furthermore, data to support that *C. trachomatis* infections have negative effects on male fertility, as well as a possible tumor promoting/initiating role of *C. trachomatis* in ovarian tumor pathogenesis, will be presented.

*Chlamydia trachomatis*

The effects of infection with *C. trachomatis* were first described in ancient Chinese writing in the Ebers papyrus (1500 B.C.) as trachoma of the eye. The name “trachoma” was first introduced in A.D. 60 and referred to the “roughness of the conjunctiva” that characterizes the ocular disease. The disease eventually became endemic but during the last century, the disease has disappeared from many parts of the world. The disappearance has been attributed to improvements in the standard of living and of hygienic practices. In hot, dry climates it still persists, and is a major cause of blindness in developing countries.

The developmental cycle, which is the unifying property that defines the genus, was first clearly described for the psittacosis agents after they had been isolated in the early 1930s. However, T’ang and colleagues (1957) in China are usually credited to have been the first to isolate the trachoma agent, which was at that time considered to be a virus. This finding boosted research on *Chlamydia* and by 1975 *C. trachomatis* was suggested to be the most common sexually transmitted bacterial pathogen worldwide. *C. trachomatis* was by then already recognized as a very common cause of urethritis in men and cervical infection in women. In 1977 Mårdh and colleagues stated that *C. trachomatis* was a major cause of pelvic inflammatory disease (PID), and subsequent studies found that this organism could be associated with tubal factor infertility and ectopic pregnancy. It was in the early 1980’s that *C. trachomatis* was divided into two groups causing primarily either ocular disease or genital disease. Since then there has been a rapid progress in the knowledge of *C. trachomatis* and its effects and diseases in humans.

*C. trachomatis* is considered the worlds leading preventable cause of blindness, with about 6 million people blinded as a result of this disease. It is also the most common sexually transmitted bacteria in the world, with approximately 90 million new cases occurring each year. In Sweden, the incidence has raised nearly three-fold since 1997 (Figure 1).
despite intensive preventive measures such as screening, partner-tracing and information. In 2008 42 000 new C. trachomatis infections were reported. The raise in incidence is most pronounced in the ages 15 – 24 years.

Chlamydia trachomatis – the bacterium

Chlamydiaceae are obligate intracellular bacteria with a unique developmental cycle. They have been placed in their own order, Chlamydiales, with one family, Chlamydiaceae, and a single genus, Chlamydia. There has been some disagreement in the scientific community whether Chlamydia should be divided into two genera, Chlamydia and Chlamydophila, based on apparent differential clustering of the 16S rRNA gene, however this separation has not been commonly accepted. The genus Chlamydia consists of four major species, Chlamydia trachomatis, Chlamydia psittaci, Chlamydia pneumoniae, and Chlamydia pecorum.

Figure 2. Simplified dendrogram relating genital C. trachomatis (serovar D-K) to other closely related bacteria within the genus Chlamydia.
Chlamydia pneumoniae and Chlamydia pecorum (Figure 2). C. trachomatis has been divided into three biovariants (biovar): trachoma, lymphogranuloma venerum (LGV) and murine (mouse pneumonitis [MoPn] agent). The trachoma and LGV biovars are distinguished by different clinical features. LGV readily cause systemic infections and proliferate in lymph nodes, whereas growth of the trachoma biovar has been believed to be limited to columnar epithelial cells at mucosal surfaces. However, chlamydial antigen and nucleic acid is also found in macrophages and smooth muscle cells deep within the lamina propria, and electron microscopic investigation has revealed C. trachomatis elementary bodies within spermatozoa.

The trachoma biovar consists of prototypical serovariants (serovars), determined by the serological immune response, and designated by the letters A through K. The serovariants A through C give rise to trachoma of the eye, whereas serovariant D through K in adults give rise to genital manifestations and in newborns pneumonia and conjunctivitis.

Developmental cycle

The developmental cycle of Chlamydia consists of a small (0.3µm) extracellular, infectious elementary body (EB), and a larger (1µm) dividing intracellular reticulate body (RB) (Figure 3). The EB has an osmotically stable and poorly permeable cell envelope and a much reduced surface area compared to the RB. The EB are also metabolically inactive, but have the capability to recognize and enter the host cell, and to reorganize and grow 30-fold in volume to the division-capable RB form. The intracellular cycle all takes place in an inclusion, a membrane-limited vacuole. Within a few hours after inclusion, EBs differentiate into the larger, metabolically active RBs. As the chlamydiae multiply, the inclusion increases in size to accommodate the multiplying bacteria, which eventually turn into EBs that accumulate within the inclusion.

**Figure 3. Chlamydia developmental cycle.**
The RBs continue to multiply until the cell lyses at 40 to 48 hours (C. trachomatis) postinfection, and the infectious EBs are released.

Persistence
Persistence of infection is the continuous presence of viable but non-infectious and non-cultivable bacteria.13 Today there is abundant evidence that chlamydiae are capable of causing enduring infections for months and years. Less is known in which developmental form chlamydiae survive long-term within the body, the susceptibility of such forms to antibiotic treatment, or the role of persistent chlamydial infection in disease. In vitro studies have shown that chlamydiae might have an abnormal development after exposure to antibiotics, developing aberrant RBs that enlarge, but resume normal developmental cycle after withdrawal of the antibiotic14 (Figure 3). Exposure to a nutrient depleted environment has shown to give similar results, as well as cytokines, particularly gamma interferon, monocyte infection, continuous infection,15 co-infection with herpes simplex virus type 2 (HSV-2)13 or sustained antibiotic treatment.16 In vivo, chlamydial DNA and antigen have been detected in culture negative subjects in tubal, ovarian and endometrial tissues as well as in prostatic tissue and semen samples.17-19 Morphologically aberrant chlamydial forms resembling those observed in vitro have been visualized by electron microscopy.20 Chlamydial RNA has been detected in the absence of cultivability in experimental trachoma of primates21 as well as in synovial biopsy samples of patients with reactive arthritis or Reiter’s syndrome,22 and in the fallopian tubes of seven women with ectopic pregnancy who were DNA positive for C. trachomatis.23 Since RNA is highly labile this indicates viable, metabolically active, but non-cultivable organisms. In animal infection models mice infected with either C. trachomatis24, 25 or C. pneumoniae,26 infections that had become asymptomatic, reactivated after immunosuppression with cortisone or cyclophosphamide. Similarly, topical therapy with corticosteroids of inclusion conjunctivitis has long been considered to lead to flare-up of trachoma disease, presumably because local immunosuppression resulted in the reactivation of clinically inapparent infection.27

Several studies addresses the question of persistent infections in the female genital tract, with a maximum follow-up period of five years (for review see Golden, 2000).28 The persistence rate varies between 29 and 87% depending on length of follow-up period, subjects included, detection method, test specimen etc. In a more recent study the persistence rate was 55% at one year,29 and in another 46% at 1 year, 18% at 2 years and 6% at 4 years.30 A C. trachomatis serovar-specific analysis was done and 53 out of 55 women were found to be infected by the same serovar at all occasions indicating persistence rather than re-infection. Oral contraceptive pill use and older age at first sexual intercourse was associated with increased clearance rate. In men, the duration of the follow-up period has lasted up to six months. Eight of nine (89%) men eligible for follow-up were still C. trachomatis positive by PCR testing in a urine specimen after six months.31

Chlamydial Heat Shock Proteins
Heat Shock Proteins (HSPs) are a group of highly conserved cellular proteins that acts as chaperones, with a key role in intracellular folding and refolding, assembly, and translocation of proteins. The expression of HSPs was initially found to be elevated in reaction to heat stress but is also expressed as well in reaction to proteolytic, mechanical or chemical stress.32 There are four main groups of HSPs based on their molecular weights: HSP90, HSP70, HSP60 and the small HSPs. During persistent infection the HSP60 production is up-regulated while the production of other proteins is down-regulated.13, 33-34
Chlamydial HSP60 is suggested to inhibit the apoptotic pathway of the host-cell supporting persistence of chlamydial infection.\textsuperscript{35} Human and chlamydial HSP60 share an approximately 50\% amino acid homology\textsuperscript{36} and despite this homology, chlamydial HSP60s are highly immunogenic and are the HSPs most extensively studied in relation to infertility. The humoral immune response to cHSP60 is in several studies associated with tubal damage and subsequent infertility.\textsuperscript{37-43} An autoimmune cross-reaction between human and chlamydial HSP60, making the woman’s immune system attack autologous HSP60, or a delayed hypersensitivity reaction, is suggested to be the mechanism for the inflammation and scarring of the tubes.\textsuperscript{36} More recent research have demonstrated that chlamydia-infected cells produce pro-inflammatory chemokines, cytokines, growth factors and other cellular modulators, sufficient to account chronic and intense inflammation and the promotion of cellular proliferation, tissue remodeling and scarring in itself.\textsuperscript{32, 44}

It is also suggested that chronic infection and inflammation, and inhibition of apoptosis by cHSP60, might have tumor promoting/initiating effects, increasing the risk for genital cancers such as cervical cancer.\textsuperscript{45} Furthermore, epithelial ovarian cancer is also suggested to be associated with \textit{C. trachomatis} infections.\textsuperscript{46, 47}

Clinical manifestations of genital \textit{C. trachomatis}

Up to as many as 85 to 90 percent of \textit{C. trachomatis} infections in men and women are asymptomatic\textsuperscript{48, 49} and can persist for several months and years. The complications encountered by the female are many different such as urethritis, Bartholinitis, cervicitis, endometritis, pelvic inflammatory disease (PID) sometimes with intraabdominal spread causing periappendiciditis and perihepatitis, and in rare cases proctitis. Symptoms range from pain when urinating to lower abdominal pain, modest fever and adnexal and uterine tenderness on pelvic examination, but often there is only a midcycle bleeding or no symptoms at all. Late sequels are infertility, ectopic pregnancy, chronic pelvic pain and probably also uterine cervix squamous cell carcinoma.\textsuperscript{50}

In men the spectrum of disease covers urethritis, prostatitis, orchitis and epididymitis.\textsuperscript{51} The role of \textit{C. trachomatis} in male infertility is a matter of debate and will be discussed more extensively in a separate section (\textit{C. trachomatis and male infertility}). Arthritis and conjunctivitis are described in both women and men, and among those who practice anal intercourse also proctitis.\textsuperscript{52} In the neonate, vertical transmission might cause conjunctivitis or neonatal pneumonia. A very early onset of disease in some cases, suggests that the infection may start as an intrauterine chlamydial infection.\textsuperscript{53}

\textbf{\textit{C. trachomatis} diagnostic methods}

\textbf{Test specimen}

To get valid test information it is important to have the right test specimen, but in \textit{C. trachomatis} diagnosis this is not always easily achieved. The infection might be localized in the endometrium, fallopian tubes, ovaries or the prostatic tissue\textsuperscript{18} which are not easily accessible, and it requires invasive procedures to get tissue samples from these locations. The common routine diagnosis involves a first void urine sample or a cervical or urethral swab. Lately vaginal swabs have been introduced with good accuracy for detection of lower genital tract infections.\textsuperscript{54} However, \textit{C. trachomatis} antigen or DNA has been found in endometrium, fallopian tubes, ovaries, semen or prostatic tissue without being able to detect any bacteria in urine specimens or cervical secretions.\textsuperscript{18} Chlamydia serology, utilizing a blood sample, might in some instances be an alternative diagnostic method with an easily accessible test specimen giving information on the immune reaction to a chlamydial infection at any site of the body.
Culture
Culture was for many years the only method available for the diagnosis of *C. trachomatis*, but has in the light of newer methods such as nucleic acid amplification tests (NAAT) been abandoned for routine diagnosis in large-scale laboratories. The sensitivity is approximately 60-70% which makes it a poor diagnostic tool, but the specificity is 100%.\(^{55}\) In brief, clinical material is inoculated onto tissue culture cells, cultured for three days and thereafter chlamydial inclusions are detected by staining with iodine or Giemsa stain, fluorochrome-labelled poly- or mono-clonal antibody or by immunohistochemistry. There are many different conditions that can reduce the sensitivity of culture such as type of clinic, how well-trained the health care providers are, and the quality of the specimens they collect, the transport systems being used, the laboratory tissue culture system, the type of stain being used, the speed of centrifugation, etc.

Immunofluorescence (IF) and antigen detection enzyme immunoassays (EIA)
The key to this entire process is the ability to visualize an antibody attached to an antigen. The direct immunofluorescence method uses a fluorescent dye that is covalently attached to the antibody. When a light illuminates the fluorescent dye, it absorbs the light and emits a different color light which is visible to the investigator and can be photographed. This was the first method not dependent on viable *C. trachomatis*, but had sensitivities and specificities lower than culture. In the early 1980s, monoclonal antibody technology made the immunofluorescence technique more specific and led to the elucidation of the relationship between the newly discovered major outer membrane proteins (MOMP) and the serotypes of *C. trachomatis*. In enzyme immunoassays, the attached enzyme-tagged antibody is detected by adding a substrate indicator that produces a color reaction. The optical density of the enzyme is read by a spectrophotometer.

Nucleic acid amplification assays – NAATs
NAATs are widely used for *C. trachomatis* diagnosis today and have proven to be more sensitive and specific than previous diagnostic tests (culture, IF and EIA) because they don’t need viable chlamydiae (more tolerant to transports) and due to the amplification process. A further advantage is that they can be used in non-invasive specimens such as a first-void urine or vaginal swabs with nearly identical sensitivity and specificity to those in cervical or urethral samples.\(^{56}\) Principally NAATs amplify either a) the target nucleic acid, DNA (polymerase chain reaction, PCR; strand displacement assay, SDA) or ribosomal RNA (rRNA) (transcription mediated amplification, TMA); or b) the probe after it has annealed to the target nucleic acid (ligase chain reaction, LCR). The major targets for amplification based tests against *C. trachomatis* are generally multiple-copy gene products, such as the cryptic chlamydial plasmid (PCR, LCR, SDA) which is present in EBs with 7 to 10 copies, or rRNA (TMA) which may have several thousands of copies per bacterial cell. The high number of target copies in TMA might theoretically be advantageous with respect to sensitivity.

The first step in the PCR process is denaturation of the DNA double stranded cryptic plasmid by heating, followed by the annealing of a specific primer. Thereafter synthesis of new DNA in a polymerase dependent process takes place where after the cycle starts over again with denaturation. These steps occur at different temperature optima, and by thermal cycling multiple copies, also called amplicons, of the target sequence are produced. The amplicons are detected by peroxidase labeled oligonucleotide probes complementary to the amplicon, which will give a colorimetric reaction when peroxidase substrate is added.
In TMA, a primer binds to an rRNA target, a DNA copy is synthesized, a second primer binds to the new DNA copy and yet another DNA copy is made. Thereafter, RNA polymerase initiates transcription of 100-1000 copies of RNA amplicons from which new DNA copies can be created, and finally double-stranded DNA. The cycle will be repeated over and over resulting in a billion-fold amplification. The amplicons are detected by adding an acridium ester labeled DNA probe that will emit light, detected by a luminometer, from hybridized probes.

Other NAATs have a principally similar way of working with a gene probe that attaches to a target gene, amplification and detection.

Specificities and sensitivities among the different NAATs are reported to be similar ranging from 95% to 100% for specificity and 80% to 93% for sensitivity.\textsuperscript{56, 57} Different specimens can in some instances affect sensitivity due to inhibitors to the amplification process. In commercial kits there are usually several different procedures to avoid inhibition.

**Serology**

The method of measuring antibodies to a microorganism in serum is widely used for research purposes to compare sample populations for prior or current exposure to an infection. There are, for example, a large number of studies which show that women with tubal factor infertility have a higher prevalence of antibodies to \textit{C. trachomatis} than fertile women.\textsuperscript{58} Regarding \textit{C. pneumoniae}, associations of serum antibodies with atherosclerosis and heart disease are being evaluated.\textsuperscript{59-62} Sensitivity and specificity for serological tests, as for all diagnostic methods, are dependent on how accurate the method detects serum antibodies and what the serological method is compared to. Concerning \textit{C. trachomatis} and infertility, the serological methods are often compared as to how well they can detect tubal pathology. However, \textit{C. trachomatis} is not the only factor that gives rise to tubal pathology. Inherently, sensitivity for the disease one wants to detect will decrease. On the other hand all \textit{C. trachomatis} infections causing antibody production do not lead to tubal factor infertility (TFI), hence specificity is reduced.

Several commercial methods are today available, among them the MIF test, once considered the “golden standard” for \textit{C. trachomatis} serology, and the ELISA method. The draw-back of the MIF-test is that it is laborious. The MIF test uses chlamydial EBs treated in a process to remove the group specific lipopolysaccharide (LPS) of the outer membrane. The residual major outer membrane protein (MOMP) has species and serotype specific antigens and constitutes approximately 60% of the organism’s outer membrane. The MIF method used in this thesis utilizes MOMP from eight serotypes of \textit{C. trachomatis}, D-K, which are known to cause genital disease. The EBs are attached to a slide to which patient sera is added and incubated. Thereafter the slide is washed to remove unbound serum antibodies. In the next stage the slide is incubated with fluorescein-labeled antibodies that react with bound human IgG or IgA. Afterwards the slide is washed, dried, mounted and examined using fluorescence microscopy. Positive reactions appear as bright apple-green fluorescent dots/EBs (specific fluorescence) (Figure 4), and can be distinguished from non-specific fluorescence, which is a homogenous green coloring of the slide. Semi-quantitative endpoint titers are obtained by testing serial dilutions of positive specimens.

The principle of the ELISA method is similar but with a different detection system. In the ELISA method detecting cHSP60 IgG antibodies, a plate is covered with recombinant cHSP60 proteins from genital \textit{C. trachomatis}. Antibodies from the serum specimen that are directed towards cHSP60 bind to the antigen. Thereafter peroxidise-conjugated anti-human
IgG antibodies bind to the cHSP60 bound IgG antibodies. Finally a substrate for the peroxidase is added and the light absorption is read photometrically.

**Effect of antibiotic treatment**

Doxycycline 100mg twice daily for 7 days, or a single dose of azithromycin 1g, are the most rigorously investigated treatment regimens for uncomplicated chlamydial infections, but doxycycline 200 mg on the first day with 6-9 subsequent doses of 100 mg daily is also studied. All regimens have microbial cure rates of >95% at 2-5 week follow up with culture, immunofluorescence or, lately, NAAT. When patients are followed up for longer periods of time following treatment, and tested with NAATs, more than 10% will be chlamydia positive on retesting. This has been considered a result of re-infection but there is now emerging evidence that this may be the result of re-emergence of persistent latent (non-detectable) infection as well as re-infection. When patients are followed up for longer periods of time following treatment, and tested with NAATs, more than 10% will be chlamydia positive on retesting. This has been considered a result of re-infection.

Figure 4. Fluorescein-labelled *C. trachomatis* elementary bodies (green dots) detected by the MIF-method. *MIF*, micro immuno fluorescence (Reprinted with permission from Focus Diagnostics Inc.)

By genotyping subsequent culture-positive *C. trachomatis* episodes, which were treated with doxycycline or azithromycin, in 7 women over a two to five year period, identical genotypes for the subsequent infections were detected indicating persistence. However, re-infection by the same untreated partner could not be ruled out. The women had culture negative cervical samples, however positive with ligase chain reaction (LCR), in between the positive cultures, supporting that *C. trachomatis* bacteria may not have been eradicated by the antibiotic regimen. Similarly, an 8% infection rate three to six months after treatment with azithromycin, in women reporting no sexual intercourse after initial treatment, has been found.

Inflammation, scaring and fibrosis are the main pathological findings in the upper genit al tract after chlamydial infection, causing tubal damage, ectopic pregnancy and infertility. In one experimental study on macaques, there was a significant difference both in eradicating *C. trachomatis* and preventing inflammatory changes after treatment with azithromycin once daily for seven days, compared to doxycycline once daily for 14 days, or placebo. In clinically apparent salpingitis and PID, anaerobic bacteria are also believed to be involved. This has resulted in the wide application of metronidazole in addition to a chlamydia specific drug.
Subfertility/infertility
Definition and prevalence
Subfertility/infertility is an important medical and social problem in both magnitude and impact on well-being. An often used definition is that the couple is regarded subfertile after one year of unprotected sexual intercourse without conception. According to the definition by the World Health Organization (WHO) 2 years of unprotected sexual intercourse is required. Infertility is the most common used term for this condition, however in fact refers to a couple that can not at all achieve pregnancy. Infertility affects approximately 5-26% of couples in the reproductive age-group, a figure that is fairly similar between less and more developed countries. An estimated 70 million couples are at the present experiencing infertility. This figure could, however, rise in the near future as increasing numbers of women delay childbearing, resulting in decreased oocyte quality, raised chance of exposure to sexually transmitted diseases, and a secular decline in sperm counts. The causes of infertility are in slightly more than 1/3 due to female factors, in 1/3 male factors and in 1/3 there is a combination of male and female factors. In 5-10% a cause can not be established despite thorough investigation.

Psychosocial aspects
For many couples, the inability to bear children is a tragedy. There is both a biological and social loss that to most people is shocking and often leads to a psychological crisis following the well-known traumatic crisis, however often more prolonged. Childless couples are also excluded from taking leading roles in important family events such as birthdays, christenings, confirmations and weddings of their children. Even though infertility is a common problem to the couple, the man and the woman might have different feelings and reactions, affecting their marital and social lives in different ways. A feeling of despair, anxiety, grief, lack of self-esteem, and sexual inadequacy are common in the infertility situation and does not seem to be related to socio-economic class, race or culture. It is essentially “human” to respond to childlessness even though the nature of concern may differ.

Chlamydia trachomatis and infertility
C. trachomatis and female infertility
C. trachomatis is known to cause damage to the female reproductive tract, primarily due to adhesions or obstructions of the fallopian tubes secondary to the inflammatory response. Reduced chances to achieve an intrauterine pregnancy is the result. TFI is the main cause of infertility in 10-30% of cases in developed countries and C. trachomatis is the most common causative agent to PID and TFI in the developed world, while the rate of Neisseria gonorrhoeae is low. In developing countries the proportion of tubal factor infertility among subfertile couples is up to 85% and the spectrum of causative agents somewhat different with a higher proportion of N. gonorrhoeae and genital tuberculosis. For example, in women in India with a history of genital tuberculosis, the rate of infertility was 58%, and among women with TFI the incidence of genital tuberculosis was 41%.

There is today plenty of evidence that C. trachomatis serum antibodies are associated with tubal factor infertility (TFI), an association that was first discovered by Punnonen and co-workers. Repeated episodes of pelvic inflammatory disease, as well as the severity of PID, increase the risk for ectopic pregnancy and infertility. Since it is widely accepted that there is an association of serum C. trachomatis IgG with TFI, the research has shifted towards how clinically useful C. trachomatis antibody testing is in the infertility work-up, but also if IgA and eHSP60 IgG antibodies has any additional value in diagnosing TFI.
In-vitro fertilization was originally designed to overcome and by-pass damaged tubes\(^9\) and TFI has ever since the introduction of the method constituted one of the main infertility causes of couples receiving IVF treatment.\(^9\) However, TFI is in itself found to be associated with a poor prognosis of IVF treatment compared with other infertility diagnoses, particularly when a fallopian tube dilated by fluid (hydrosalpinx) is present.\(^9\) How the negative effect is mediated is still unknown\(^9\) but salpingectomy of ultra-sound visible hydrosalpinx prior to IVF is shown to increase pregnancy rates.\(^9\)

C. trachomatis antibodies are also proposed to be associated with a reduced success rate when IVF treatment is carried out. Chlamydial HSP60 IgG\(^9\) or IgA\(^1\) antibodies in follicular fluid, or cHSP60 IgA antibodies in the cervix,\(^1\) were associated with a reduced implantation rate after IVF treatment. Suggested mechanisms are either that cHSP60 IgG antibodies indicate a persistent infection causing an inflammatory reaction undermining embryo implantation. Alternatively, cHSP60 IgG antibodies cross-react with human HSP60, expressed at vital stages of embryogenesis,\(^3\) and may induce destruction of the embryo.

In recent years, the role of genetic traits of the host immune system for the development of tubal pathology in C. trachomatis genital infections has received increasing attention. Several markers have been identified such as HLA-A31 that has been correlated with acute chlamydial pelvic inflammatory disease,\(^1\) variants at IL10 (encoding interleukin-10) promoter positions with higher IL-10 concentrations in cervical secretions associated with recurrent Chlamydia infection,\(^3\) HLA-DQ alleles combined with IL10 promoter polymorphism associated with TFI,\(^1\) and multiple SNPs (single nucleotide polymorphisms) in the pattern recognition receptors (PRRs) involved in sensing bacterial components that are associated with tubal pathology following a C. trachomatis infection.\(^1\)

C. trachomatis and male infertility

C. trachomatis is a well-known pathogen causing damage to the female reproductive tract leading to reduced fertility. Less is known about the possible effects on fertility in men apart from causing postinflammatory strictures to the epididymis/vas, in rare cases, leading to a mechanical hinder for the sperm to reach the female reproductive tract. There are several previous reports on C. trachomatis antibodies in serum or semen in men in relation to reduced pregnancy rates, and impaired semen characteristics, some showing an association\(^1\) while others did not.\(^1\) Sexual transmission of C. trachomatis bacteria to the female partner, thereby causing damage to the female reproductive tract leading to reduced pregnancy rates, has been one possible explanation as to why C. trachomatis antibodies in the man are related to reduced pregnancy rates. Lately, C. trachomatis bacteria in semen has been associated with impaired semen characteristics,\(^3\) and in in vitro, studies co-incubating human sperm with C. trachomatis, a direct negative effect of C. trachomatis on the spermatozoa has been found.\(^3\) Reduced motility, vitality, blunted acrosome reaction and sperm DNA fragmentation are among the effects reported.

Ovarian tumors

Prevalence and risk factors

Ovarian cancer is the sixth most common cancer among females and the most lethal gynaecologic malignancy with a 16-51% five year survival rate globally.\(^3\) The incidence is higher in the developed world compared to the developing countries (Figure 5). No firm conclusions are established on the etiology of ovarian cancer, while 5-10% is attributable to genetic predisposition.\(^3\) Nulliparity, in-
fertility, hormone replacement therapy, endometriosis, high BMI and selected aspects of diet are established or proposed risk factors while child-bearing, oral contraceptive pill use, tubal ligation, hysterectomy and breastfeeding are protective factors. Incessant ovulation, “gonadotropin stimulation” and “retrograde transportation” hypotheses have been proposed to explain the etiology of epithelial ovarian cancer. However, the high risk-reduction by for example one pregnancy, a few years of oral contraceptive pill use, or a late menopause does not correspond to the small reduction in numbers of ovulations or the short duration of diminished levels of gonadotropins. Apparently, there must be some other biological mechanisms.

Ovarian tumor biology
According to the cell types from which ovarian tumors are assumed to originate, they are divided into three major groups: surface epithelial-stromal tumors, germ cell tumors and sex cord-stromal tumors. The surface epithelial-stromal tumors account for approximately 60% of all ovarian tumors and 90% of malignant ovarian tumors and are commonly addressed as epithelial ovarian cancer (EOC). Fifteen percent of epithelial ovarian neoplasms exhibit exuberant cellular proliferation but no invasive behavior and are accordingly classified as borderline ovarian tumors (BOT), sometimes also called “of low malignant potential” or “atypically proliferating”. Epithelial ovarian tumors can be subdivided into five major subtypes designated as follows: serous, mucinous, endometrioid, clear cell, and transitional cell (or Brenner type). Highly malignant epithelial tumors lacking specific differentiation are classified as undifferentiated. BOT are mostly of serous or mucinous subtypes.

Serous tumors are tumors formed by cells that resemble those of the internal lining of the fallopian tube, are often cystic, and the malignant serous tumors are in a majority of cases widely disseminated at the time of diagnosis. Serous or mucinous tumors identical to those in the ovary may arise in multiple locations within the pelvic region and the abdominal cavity. When there are simultaneous

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**Figure 5.** Incidence of ovarian cancer (age-standardized) per 100 000 women and year (Sankaranarayanan, R. and Ferlay, J., Best Pract Res Clin Obstet Gynaecol, 2006).
INTRODUCTION

In 2004 Shih and Kurman first proposed a new model for the pathogenesis of ovarian tumors dividing the tumors into two groups, type I and type II, with different pathogenetic pathways. The model is based on clinical, pathological and molecular genetic studies. Type I tumors are slow growing, generally confined to the ovary at diagnosis and develop from well-defined precursor lesions. They include low-grade micropapillary serous carcinoma, mucinous, endometrioid and clear-cell carcinomas. Type II tumors, approximately 75% of ovarian carcinomas, are on the other hand rapidly growing, highly aggressive neoplasms. They lack well-recognized precursor lesions and are almost always discovered after peritoneal or serosal involvement has taken place. Type II tumors include moderate or high-grade serous carcinomas, malignant mixed mesodermal tumors, and undifferentiated carcinomas. Type I tumors are genetically stable and have mutations in a number of different genes including KRAS, BRAF, PTEN and beta-catenin, while type II tumors are characterized by p53 mutations with p53 protein over-expression and a high level of genetic instability.

The precursor lesions of the type I tumor group are cystadenomas, serous borderline tumors, mucinous borderline tumors and endometriosis, which are often associated with their corresponding carcinoma. Tubal intraepithelial carcinoma (TIC) is an emerging candidate precursor for the type II tumor group, and has been found associated with high-grade serous ovarian and pelvic carcinomas. The identical p53 mutations have been identified in TIC and serous carcinomas of the same patient, and also in adjacent “p53 signatures”. p53 signatures are areas, preferably in benign mucosa in the tubal fimbriae, with strong p53 immunostaining, targeting the secretory cells.

Chronic infection and inflammation in the pathogenesis of ovarian tumors

Other types of cancer

Microbes causing chronic inflammatory disease have in the last decade become increasingly investigated as possible cancer initiators/promoters and infectious etiologies have been identified for some cancers. Helicobacter pylori colonization of the stomach may lead to gastric cancer, particular subtypes of HPV may initiate cervical cancer and chronic inflammation induced by Hepatitis B and C virus infections can cause liver cancer. C. trachomatis is considered a cofactor in cervical cancer and is primarily associated with squamous cell carcinoma of the cervix.
**C. trachomatis** and ovarian cancer

The role of persistent infection, leading to chronic inflammation, in the pathogenesis of ovarian cancer has received very little consideration, although a history of pelvic inflammatory disease (PID), adjusted for age, parity, duration of oral contraceptive use and infertility, is in a case-control study associated with higher risk for ovarian cancer.147 **C. trachomatis**, the most common cause of PID in the developed world,82-84 is one of the genital infectious agents that has been addressed as a possible tumor initiator/promoter of the ovaries.148 It is recently hypothesized that the origin of ovarian cancer might be the junction between the oviductal epithelium and the ovarian surface epithelium, an area of transitional epithelia with increased susceptibility to neoplastic progression.149 In parallel with cervical cancer occurring at the squamos columnar junction and gastroesophagic cancer occurring at the esophagogastric junction, this might be a conflict area where chronic infection with for example **C. trachomatis** might cause cellular damage and proliferation, leading to cancer development.150 This is also the area where the type II tumors of the ovary are suggested to originate.

Persistence of infections are thought to play a key role in the possible association between bacteria and cancer, and there is evidence that **C. trachomatis** can become persistent, and express high levels of chlamydial Heat Shock Protein 60 (cHSP60) during persistent infections.35, 151 Chlamydial HSP60 is suggested to have an anti apoptotic effect developed to facilitate the survival of the bacteria within the host.35 The inflammatory reaction in chronic infection causes production of cytotoxic substances that might cause damage to the DNA. Increased cell-turnover is also part of the inflammatory reaction, while cHSP60, by having an anti-apoptotic effect, facilitates the survival of DNA-damaged cells leading to increased risk for cancer initiation.46 As **C. trachomatis** is especially adept at maintaining a long-term relationship with its hosts, modulating and evading the immune system, and is shown to cause infertility (which in itself is a suggested risk factor for ovarian cancer), there is a possibility that **C. trachomatis** infections might play a role in carcinogenesis of the ovary.15, 47, 152

**Previous studies**

Ness et al. have found an association of **C. trachomatis** IgG antibodies with ovarian cancer, and a non-significant monotonic trend towards increased ovarian cancer risk associated with cHSP60-1 IgG antibodies, but no associations of cHSP60-2 or cHSP60-3 IgG antibodies.153 However, in a second study no associations of **C. trachomatis** antibodies and ovarian cancer risk were found,154 rather a negative association in the younger age groups. Neither did Wong et al. find any associations of serum IgG, IgA or IgM antibodies to **Chlamydia spp.** However, a serological method detecting antibodies to all **Chlamydia** species including **C. pneumoniae** was used,155 which might have diluted a possible association.

**Other genital infections as possible tumor promoters/initiators**

**Mycoplasma genitalium** (*M. genitalium*), the smallest self-replicating organism known, is another sexually transmitted microorganism that in recent years has been associated with PID, TFI156, 157 and infertility.158 In vitro, *M. genitalium* can cause inflammatory changes of the epithelium of the human fallopian tubes.159 Similar to **C. trachomatis** infections, disease caused by *M. genitalium* is often asymptomatic, or reveals only mild to moderate symptoms and it often remains undetected.160 *M. genitalium* is not previously studied in relation to ovarian cancer, however *Mycoplasma* DNA (PCR targeting DNA from 15 different species) has been found in 59% of ovarian cancer tissues.161 Other *Mycoplasma* species have been associated with various cancers such as renal cell carcinoma162 and
cervical intraepithelial neoplasia (CIN), and *Mycoplasma hyorhinis* with gastric and colon cancer.

The incidence of *N. gonorrhoeae* in Sweden is considerably lower than for *C. trachomatis* and has from the 1980’s showed a steady decline with an incidence of 2.4 per 100 000 inhabitants in 1996. From 1997 the incidence has risen slightly and was in 2008 7.8 per 100 000 (compared with 454.1 per 100 000 for *C. trachomatis*). *N. gonorrhoeae* is known to cause PID and TFI and the infection remains asymptomatic in up to 80% of women. In men, *N. gonorrhoeae* is linked to prostate cancer in several meta-analyses. However, it is not previously studied in relation to ovarian cancer.

HPV is a well established cause of cervical cancer and the high-risk types are present in more than 95% of cervical cancer biopsies. Regarding HPV in ovarian tissues there are, today, a great number of reports focusing on a possible tumor initiating/promoting role of the virus. Some have detected HPV in ovarian carcinoma tissues while others have not. Serum HPV antibodies were not associated with ovarian cancer in one study.

Polyomaviruses were originally discovered in 1953 but not until 1971 the identification of two new polyoma viruses infecting humans were reported. BK virus (BKV) was isolated from the urine of a renal transplant patient with the initials B.K., while JC virus (JCV) was first discovered in the brain of a Hodgkin lymphoma patient who suffered from progressive multifocal leukoencephalopathy and whose initials were J.C. The polyoma viruses BKV and JCV have been found in several different human tumors (urinary tract, kidney, bone, pancreatic islet, a wide variety of brain tissues and skin tumors – Merkel cell carcinoma) but a causal link has not been established. In vitro BKV and JCV are known to cause transformation of human cells, and they can induce different types of tumors in several rodent species. The presence of these viruses in ovarian tumors is not previously explored. Recently, findings of a new polyomavirus in Merkel Cell carcinomas are strongly suggestive of an oncogenic role of the virus.
Aims

The overall aim of this thesis was to further investigate the effects of *Chlamydia trachomatis* infection on fertility in men as well as in women, and *Chlamydia trachomatis* as a possible risk factor for developing ovarian cancer. The specific questions that needed to be answered were:

- Does a *C. trachomatis* infection in the man or the woman, detected by serum *C. trachomatis* antibodies, affect the chances of the couple to achieve pregnancy and have a child?
- Can a *C. trachomatis* infection exert a direct effect on male fertility, and do semen characteristics differ between serum *C. trachomatis* antibody positive and negative men of infertile couples?
- Is infection with *C. trachomatis*, detected by plasma antibodies, associated with epithelial ovarian cancer or borderline ovarian tumors?
- Can *C. trachomatis* bacteria be detected in ovarian tissues from women with epithelial ovarian cancer, borderline ovarian tumors or benign conditions?
- Are *M. genitalium* plasma antibodies associated with ovarian tumors, or can *M. genitalium*, *N. gonorrhoeae*, HPV or polyoma viruses BKV and JCV, be detected in ovarian tissues?
Materials and Methods

Ethics
All studies were approved of by the Human Ethics Committee of the Medical Faculty, Umeå University, Sweden. Women and men were included after informed consent and the investigations and treatments were uninfluenced by the study. Screening for *C. trachomatis* among infertile couples may however, if positive, raise questions about unfaithfulness and extramarital sexual activity that could be difficult to handle within the relationship. Professional counseling was offered to all. No tissue in excess to what was clinically relevant was removed. An inconvenience to the women and men included was an extra blood sample for some. Control persons from the Antenatal Care program and the Northern Sweden Health and Disease cohort had previously donated blood for research purposes.

Papers I and II: Infertility study
Paper I was a prospective cohort study. Associations of *C. trachomatis* IgG with diagnosis, pregnancy rate and pregnancy outcome were analyzed comparing exposed and non-exposed within the cohort. The prevalence of *C. trachomatis* IgG antibodies in the cohort of infertile women was compared to the prevalence in spontaneously pregnant women. Paper II was based on the same cohort. However, additional serum *C. trachomatis* antibodies were analyzed retrospectively using frozen serum samples, and details of the semen characteristics were reviewed.

Study population
During October 1997 to February 2001, 244 consecutive couples attending the gynecological outpatient clinic at the Department of Obstetrics and Gynecology, University Hospital of Northern Sweden, due to infertility (one year of unprotected sexual intercourse without conception) were included (Figure 6). Blood was drawn upon their first visit and tested for *C. trachomatis* IgG antibodies, while the testing of *C. trachomatis* IgA and IgM, and cHSP60 IgG, antibodies was done retrospectively. Both partners were also tested for HIV and hepatitis B and C, and the women for rubella immunity and for ovulation using a mid-luteal serum progesterone according to routine infertility work-up. Semen analysis of the man (according to the World Health Organization as modified by NAFA/ESHRE) was undertaken, described in detail in paper II. If either one of the partners had *C. trachomatis* IgG antibodies, a first-void urine sample for detection of *C. trachomatis* DNA was collected from both. If at least one of the partners of the couple had detectable *C. trachomatis* DNA, they were both treated with doxycycline 100 mg x 2 the first day and thereafter doxycycline 100 mg x 1 for 9 more days.

Clinical investigation
One to three months later, when the test results were available, a consultation according to routine infertility work-up was conducted,
including clinical examination and ultrasonography. Thereafter further investigations such as hysterosalpingosonography (HSS), laparoscopy or additional hormonal assays were planned. The couples were advised about treatment according to the findings. If both tubes were patent without dilation on HSS, no further tubal investigation was carried out. Else, a laparoscopy with chromoperturbation was planned. The occurrence of an interval spontaneous pregnancy before tubal assessment was considered a surrogate marker for the absence of tubal pathology. However minor degrees of tubal pathology may have been present.

In paper I, the clinical diagnosis of male factor, as reported in the medical records, was analyzed. In paper II, details of the semen...
characteristics were included in the analyses. Furthermore, male factor was defined as a sperm concentration below 20 millions/mL\(^{195}\) and/or teratozoospermia index (TZI) >1.79 and/or proportion motile spermatozoa <40%. Hence, a reclassification of male factor had to be done.

**Follow-up**

After a follow-up period of 14-54 months (mean 37 months) the medical records of all couples included were studied with respect to findings at HSS and laparoscopy, clinical diagnoses, treatments and reported pregnancies. Pregnancy outcome was classified as either past 28 weeks of gestation, spontaneous abortion or ectopic pregnancy. Treatment related pregnancies were screened by ultrasound soon after a positive pregnancy test, and all pregnancies were routinely screened by ultrasound at gestation week 15-17. Couples that had not achieved pregnancy by the time the medical records were reviewed, were subjected to a structured telephone interview (carried out by A.I.). They were asked about treatments not found in the medical records (some couples were treated elsewhere), and pregnancies and pregnancy outcome that had not previously been recorded. Pregnancy data were available in 238 of 244 (97.5%) of the couples.

**Fertile controls**

To compare the prevalence of *C. trachomatis* IgG antibodies in women from infertile couples with the prevalence in proven fertile women (paper I), banked sera from women attending the Antenatal Care program at the local health care centers, caring for women in the same geographic area as the women attending the gynecologic outpatient clinic, were analyzed (n=244). Women were matched with respect to age (birth year) and the year when blood samples were collected. Only women with spontaneous pregnancies (as recorded in the medical records) were included.

**Papers III and IV: Ovarian tumor study**

**Study population**

Paper III was a case-control study while paper IV was a cross-sectional study.

From 1993 to 2001, 430 women who underwent surgery at the Department of Obstetrics and Gynecology, University Hospital of Northern Sweden, Umeå, Sweden, due to suspected ovarian pathology were included in the study after oral and written informed consent. In total, plasma samples (paper III) were available for 291 women (Figure 7), whereof in 238 cases blood was drawn in connection with surgery. For another 53 cases prospective plasma samples (maximum 5.1 years prior to diagnosis) from the Northern Sweden Health and Disease Cohort (NSHDC) were obtainable and included in the analysis. NSHDC, described in detail elsewhere,\(^{196}\) is a population based health survey cohort in Västerbotten County and serves the same population as the University Hospital of Northern Sweden regarding ovarian tumor surgery. In cases of BOT, EOC and other pelvic malignancies, control plasma samples from the NSHDC were matched with respect to age and date of plasma sampling. Control subjects were alive and without a cancer diagnosis (except non-melanoma skin cancer) at the time of diagnosis of the index case. Four controls per case were analyzed. Ovarian tissue (paper IV) was obtainable in 186 women, and also from the contralateral ovary in 126 women (Figure 7). In 128 women, both plasma and ovarian tissue were available.

**Clinical characteristics and histopathologic diagnosis**

Information on histopathologic diagnosis, according to the World Health Organization classification,\(^{197}\) as well as data on clinical characteristics, was extracted from the medical records. Histopathologic diagnoses were reviewed by a specialist in gynecologic pathology (E.L.). For paper III a reclassification ac-
According to the proposed hypothesis of a type I and a type II pathogenetic pathway, described in the Introduction section, was done. The type I tumors consisted of low-grade serous carcinomas and mucinous, endometrioid and clear cell carcinomas. The type II tumors, that were of particular interest, consisted of ovarian moderate and high-grade serous carcinomas, malignant mixed mesodermal tumors and undifferentiated carcinomas, including also serous high-grade or undifferentiated peritoneal or fallopian tube cancer since they are suggested to have a common origin.

C. trachomatis analyses

C. trachomatis serology

The principles for C. trachomatis serology are described in the Introduction section. A commercial MIF test (MRL Diagnostics/Focus, Cypress, CA, USA) was used according to the instructions of the manufacturer to detect serum C. trachomatis IgG and IgA antibodies. A weak specific immunofluorescence signal in the 1/40 (IgG) and 1/16 (IgA) dilutions were reported as positive in 1/20 and 1/8 respectively. A low cut-off value was chosen to increase the sensitivity of the test, despite the risk of a decreased specificity. This was done since the infections of interest for these studies were not the acute infections but rather

Figure 7. Study cohort originating from women who underwent surgery due to suspected ovarian pathology. Orange boxes show number of women with plasma sample obtainable, and yellow boxes the number of matched controls from the NSHDC (Northern Sweden Health and Disease Cohort) matched with respect to age and time of plasma sampling (paper III). Blue boxes show number of women with ovarian tissue obtainable (paper IV). A: Number of tissues from primarily affected ovaries. C: Number of tissues from contra lateral ovaries. All women with tissue from the contra lateral ovary had tissue obtainable from the primarily affected ovary as well.

a Number of women for whom 4 matched controls per case were obtainable.
previous, persistent or chronic infections. *C. trachomatis* IgG titers at the beginning of an acute infection raise rapidly, where after the titer levels decrease to a lower level.\textsuperscript{1} Chlamydial HSP60 IgG antibodies were analyzed with a commercial ELISA kit specific for detection of cHSP60 type 1 (cHSP60-1) IgG antibodies. Genomic studies have demonstrated that the chlamydial genome encodes three versions of HSP60, and type 1 is being expressed in the largest amounts\textsuperscript{198} and has been associated with cervical cancer.\textsuperscript{45} Cut off was defined as the mean optical density (OD) value of the negative control plus 0.350. Plasma with OD values ±10% of the cut off value were interpreted as indeterminant and were excluded from analysis.

DNA and RNA extraction

Tissue, 25-30 mg for each extraction, was cut frozen in a clean area with a sterile knife. RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) and DNA was extracted with QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. In principle, a lysis buffer is added to the tissue sample to lyse the proteins. Thereafter DNA/RNA is bound to a membrane and the residual contaminants are washed away. Finally, the purified DNA/RNA is eluted from the membrane and is ready to be used in a NAAT. Since human, as well as bacterial or viral, DNA and RNA are purified, the human β-globin gene (two copies per cell) was used as a positive extraction control.

*C. trachomatis* DNA and RNA analyses

The principles of NAATs are described in the Introduction section. For the infertility studies (paper I and II) a commercially available PCR test (COBAS AMPLICOR *C. trachomatis* test; Roche Diagnostics, Basel, Switzerland) was used to analyze the presence of *C. trachomatis* DNA in first-void urine samples. In an attempt to increase the chance of finding *C. trachomatis*, that might be present at very low loads in the ovarian tissue samples (paper IV), we chose to use a commercially available TMA test (Aptima Combo 2, Gen-Probe Inc., USA)\textsuperscript{199} for paper IV. TMA has a theoretically higher sensitivity due to the target gene, which is the rRNA present in thousands of copies in each cell. Positive and negative controls were included in each run.

The RNA-extraction, transportation and test method (paper IV) were validated by spiking ovarian tissue samples from 4 different women with 10, 100, 1000, 10 000 and 100 000 *C. trachomatis*, serovar D-K, bacteria respectively. RNA was similarly extracted, added to collection tubes, transported and analyzed with the Aptima Combo 2 test. Further, urine samples positive or negative for *C. trachomatis* with the Becton Dickinson ProbeTec (BD Diagnostics, Sparks, Massachusetts, USA) were also tested with the same RNA extraction method and Aptima Combo 2, with the expected results.

Additional analyses

**Paper III:** *M. genitalium* serology

*M. genitalium* IgG antibodies were detected in plasma samples from cases and controls using a *M. genitalium* Lipid associated membrane protein – Enzyme immuno assay (LAMP – EIA) as previously described.\textsuperscript{200}

**Paper IV:** *N. gonorrhoeae, M. genitalium*, HPV and polyoma virus BKV and JCV detection

The presence of *N. gonorrhoeae* rRNA in ovarian tissue samples was analyzed by the TMA method (Aptima Combo 2). *M. genitalium* DNA was analyzed using a *M. genitalium* realtime PCR (MgRT – PCR) method as previously described.\textsuperscript{201, 202} HPV, polyoma virus BKV and JCV, and the human beta-globin gene were detected by PCR. For further details, see paper IV.
**Statistics**

Statistical analyses were performed using the SPSS software. A majority of the data analyzed are dichotomous why Pearson Chi-square, and when the expected frequency was <5, Fisher’s exact test was used. Spearman’s rank correlation was used for calculating correlations (Paper I: *C. trachomatis* IgG titers and positive PCR test, *C. trachomatis* IgG titer levels between the partners). Mann-Whitney U test was applied to analyze continuous data not normally distributed (Paper II: semen characteristics; Paper III: Clinical characteristics with continuous data). Ordinal data were analyzed using the Mantel-Haenszel test. Relative risk and 95% confidence intervals (CI) were calculated to estimate the strength and precision of the associations (Paper II). Odds ratios (OR) and 95% confidence intervals (CI) were calculated using binary logistic regression analysis (Paper I and III), and a multivariate analysis controlling for possible confounding factors was done in papers I-III. A 2-sided *P*-value of less than 0.05 was considered significant. To minimize the risk of getting a type 1 error when making multiple comparisons, a sequentially rejective multiple tests procedure according to Bonferroni-Holm was performed (Paper I and III).
Results and Discussion

Papers I and II: Infertility study

Prevalence of *C. trachomatis* antibodies and DNA

The prevalence of serum *C. trachomatis* IgG, IgA and IgM, and cHSP60 IgG antibodies are given in Figure 8. We found a raised prevalence of serum *C. trachomatis* IgG antibodies among infertile women compared with proven fertile women (24% vs. 16%, $P = 0.02$). However, in the group of infertile women without TFI the prevalence was only 19% compared with 44% among women with TFI ($P<0.001$) (Table 1). This is in the same range as other Scandinavian studies. Similarly, the reported incidence rates in Sweden indicate higher incidence among women, at least in younger age groups, than men (Figure 1) possibly resulting in higher antibody prevalence. On the other hand, in this cohort of infertile men and women, it might reflect that women may be more prone to have a negative effect on fertility of a *C. trachomatis* infection than men, or have a more pronounced humoral immune response to a *C. trachomatis* infection. In several cross-sectional population-based studies the prevalence rates of *C. trachomatis* infection are similar in women and men.

There is abundant evidence of the negative

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Paper I: *C. trachomatis* IgG (n = 244) and DNA.

Paper II: *C. trachomatis* IgA, IgM, cHSP60 IgG (n = 226) and IgG/IgA

IgG+/IgA+ percentage refers to women and men in whom both *C. trachomatis* IgG and IgA antibodies were present.

DNA (% of IgG+): *C. trachomatis* in a first-void urine specimen was present in 11% of IgG+ women and 14% of IgG+ men, thus in 5 women and 5 men. In only one couple both partners were positive.
effects of C. trachomatis infections on female fertility, while the results of studies on the effects of C. trachomatis infection on male fertility diverge. The cohort of proven fertile women was an easy accessible control group since serum, drawn at the beginning of each pregnancy, is prospectively banked within the Northern Sweden Maternity Cohort. The C. trachomatis IgG antibody analysis of the proven fertile women was done retrospectively for paper I. There is no corresponding Biobank with blood from proven fertile men, but serum from healthy blood donors from the same time period may have served the purpose as a fertile control group. Regarding semen characteristics, men with C. trachomatis IgG and IgA antibodies were compared with men from other infertile couples who might have reduced sperm quality for other reasons. Using proven fertile men as a control group might have resulted in larger differences in semen characteristics.

C. trachomatis DNA was detected by PCR in a first-void urine sample in 5 women and 5 men. In only one couple both the man and the woman were C. trachomatis DNA positive by PCR test. In a recent study, C. trachomatis infections detected by both NAAT and culture or direct fluorescent antibody were concordant within sexual relationships in 75%. If detected by NAAT only, the concordance rate was 45%, compared to 20% in this study. The low concordance rate if detected with NAAT only might indicate lower bacterial load, lower transmissibility and persistent infections that are non-infectious. Additionally, ex-

Table 1. C. trachomatis serum antibodies in women from infertile couples (n=226), in relation to infertility diagnoses.

<table>
<thead>
<tr>
<th>Diagnoses and C. trachomatis antibodies</th>
<th>No (%) with diagnose with antibody</th>
<th>No (%) without diagnose with antibody</th>
<th>RR</th>
<th>95% CI</th>
</tr>
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<tbody>
<tr>
<td>TFI</td>
<td>45</td>
<td>181</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>20 (44)</td>
<td>35 (19)</td>
<td>2.5</td>
<td>1.5-4.2</td>
</tr>
<tr>
<td>IgA+</td>
<td>10 (22)</td>
<td>24 (13)</td>
<td>1.6</td>
<td>0.88-2.9</td>
</tr>
<tr>
<td>cHSP60 IgG+a</td>
<td>12 (27)</td>
<td>24 (14)</td>
<td>1.9</td>
<td>1.08-3.3</td>
</tr>
<tr>
<td>Male factor</td>
<td>72</td>
<td>154</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>8 (11)</td>
<td>47 (30)</td>
<td>0.39</td>
<td>0.20-0.76</td>
</tr>
<tr>
<td>IgA+</td>
<td>7 (10)</td>
<td>27 (17)</td>
<td>0.61</td>
<td>0.30-1.3</td>
</tr>
<tr>
<td>cHSP60 IgG+a</td>
<td>8 (12)</td>
<td>28 (19)</td>
<td>0.67</td>
<td>0.35-1.3</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>41</td>
<td>185</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>13 (32)</td>
<td>42 (23)</td>
<td>1.4</td>
<td>0.80-2.6</td>
</tr>
<tr>
<td>IgA+</td>
<td>7 (17)</td>
<td>27 (15)</td>
<td>1.1</td>
<td>0.56-2.5</td>
</tr>
<tr>
<td>cHSP60 IgG+a</td>
<td>5 (13)</td>
<td>31 (17)</td>
<td>0.74</td>
<td>0.31-1.8</td>
</tr>
<tr>
<td>Anovulation</td>
<td>52</td>
<td>174</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>9 (17)</td>
<td>46 (26)</td>
<td>0.65</td>
<td>0.34-1.3</td>
</tr>
<tr>
<td>IgA+</td>
<td>5 (10)</td>
<td>29 (17)</td>
<td>0.60</td>
<td>0.25-1.5</td>
</tr>
<tr>
<td>cHSP60 IgG+a</td>
<td>4 (8)</td>
<td>32 (19)</td>
<td>0.45</td>
<td>0.17-1.2</td>
</tr>
<tr>
<td>Unexplained</td>
<td>25</td>
<td>201</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>7 (28)</td>
<td>48 (24)</td>
<td>1.2</td>
<td>0.53-2.8</td>
</tr>
<tr>
<td>IgA+</td>
<td>5 (20)</td>
<td>29 (14)</td>
<td>1.4</td>
<td>0.56-3.6</td>
</tr>
<tr>
<td>cHSP60 IgG+a</td>
<td>5 (20)</td>
<td>31 (16)</td>
<td>1.3</td>
<td>0.50-3.2</td>
</tr>
</tbody>
</table>

*217 women had valid data in cHSP60 IgG analysis. 7 women had indeterminant results and were excluded from analysis. RR, relative risk (for disease if antibody positive); CI, confidence interval; TFI, tubal factor infertility.
plaining the low concordance rate in this study, the cohort consisted of infertile couples with no signs or symptoms of acute *C. trachomatis* infection. The concordance between *C. trachomatis* serum IgG in this study was higher; if the man was IgG pos the woman was also IgG pos in 59% of cases and if the woman was IgG pos the man was IgG pos in 47% of cases ($P = 0.0005$).

**C. trachomatis** antibodies and female infertility

The relative risks (RR) and 95% confidence intervals (95% CI) for the different infertility diagnoses, chance to achieve pregnancy and chance to have a baby (passed 28 weeks of gestation) if pregnant, depending on antibody status are given in table 1 and 2. *C. trachomatis* IgM antibodies are not presented in this or the following tables, since no pattern of associations were found for this antibody in either the men or the women, not even with a positive PCR test. IgM antibodies are known to reflect acute infections while the couples in this cohort were more likely to have chronic infections. Consistent with the findings of several previous studies, *C. trachomatis* IgG and chSP60 IgG were associated with an increased risk for TFI. However after a multivariate analysis, adjusting for chSP60 IgG antibodies, age, male factor infertility, male serum *C. trachomatis* IgG and chSP60 IgG antibodies, only *C. trachomatis* IgG was significantly associated with TFI. Interestingly, *C. trachomatis* IgG in the woman was negatively associated with a reduced risk of male factor infertility, probably because IgG antibodies in the woman were associated with TFI, and hence, the most probable cause for the infertility of the couple. If the woman had TFI the likelihood for a male factor infertility tended to decrease (RR = 0.57, 95% CI 0.31 - 1.1).

One might expect that the negative effect of *C. trachomatis* on female fertility would have been, at least partially, overcome by IVF treatment since it bypasses the requirement for patent fallopian tubes. However, a slightly reduced pregnancy rate was found among IVF treated couples (n = 32), and couples with TFI as their main diagnosis (n = 37) if the woman was *C. trachomatis* IgG positive (RR = 0.55, 95% CI 0.27 - 1.2, and RR = 0.56, 95% CI 0.29 - 1.05 respectively). The numbers in each group are too small to adjust for possible

---

**Table 2. C. trachomatis** serum antibodies in women from infertile couples (n=226), in relation to pregnancy rate and pregnancy outcome.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Achieved pregnancy</th>
<th>Passed 28 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (%) of pregnant with antibody</td>
<td>No (%) of not pregnant with antibody</td>
</tr>
<tr>
<td>IgG+</td>
<td>143 (21)</td>
<td>83 (30)</td>
</tr>
<tr>
<td>IgA+</td>
<td>30 (14)</td>
<td>25 (17)</td>
</tr>
<tr>
<td>cHSP60 IgG+</td>
<td>20 (15)</td>
<td>14 (19)</td>
</tr>
</tbody>
</table>

**Notes:**

- After 14-54 months (mean 37 months) of follow-up.
- 217 women had valid data in cHSP60 IgG analysis. 7 women had indeterminant results and were excluded from analysis.
- Number of women that passed 28 weeks of gestation out of the 143 women that achieved pregnancy.

**RR**, relative risk (or chance to achieve pregnancy and have a child if antibody positive); **CI**, confidence interval.
confounding by *C. trachomatis* antibodies in the male partner. In some IVF treated cohorts chlamydial antibodies were not associated with reduced pregnancy rates.\textsuperscript{208-213} In one study all *C. trachomatis* IgG positive women were treated with doxycycline prior to IVF possibly masking an association.\textsuperscript{214} In several others, *C. trachomatis* IgG antibodies have been a predictor of negative IVF outcome.\textsuperscript{215-217} Similarly, cHSP60 IgA antibodies in the cervix\textsuperscript{101} or cHSP60 IgG or IgA antibodies in follicular fluid\textsuperscript{99 100} have been associated with impaired embryo implantation rates after IVF. One possible explanation for a reduced pregnancy rate is that there still is a chronic *C. trachomatis* infection causing an inflammatory reaction which prevents successful embryo implantation. Another, is the suggested auto-immune cross-reaction of cHSP60 IgG antibodies to human HSP60 due to the structural similarities of human and chlamydial HSP60.\textsuperscript{36} Since HSP60 is expressed at unique developmental stages of embryogenesis,\textsuperscript{218} cHSP60 IgG antibodies will exert a negative effect on embryo development and implantation resulting in decreased reproductive outcome.

One way to evaluate the clinical usefulness of a diagnostic test is to estimate the positive predictive value (PPV) and the negative predictive value (NPV) which are, apart from sensitivity and specificity of the test, also dependent on the prevalence of the disease in the population of interest. In this cohort of infertile women the PPV of *C. trachomatis* IgG in diagnosing TFI was 36% and the NPV was 85%, indicating that the test alone is mediocre in discriminating between women with TFI and those without. Combining the test for *C. trachomatis* IgG antibodies with cHSP60 IgG antibodies did not improve the PPV or NPV.

*C. trachomatis* has been associated with ectopic pregnancy, preterm labor, premature rupture of the membranes (PROM) and preterm delivery.\textsuperscript{219} In this study the rate of ectopic pregnancy was somewhat higher in the IgG pos group (3/32, 9.4%) than in the negative group (3/118, 2.5%) but the numbers are too small to draw any conclusions. Since not all pregnancies were followed until term the rate of preterm labor, premature rupture of the membranes or neonatal infections could not be estimated. However, no difference in the number of antibody positive and negative women passing 28 weeks of gestation was found.

*C. trachomatis* antibodies and male infertility

The relative risks (RR) and 95% confidence intervals (95% CI) for the different infertility diagnoses, chance to achieve pregnancy and chance to have a baby (passed 28 weeks of gestation) if pregnant, depending on antibody status in the man are given in table 3 and 4. *C. trachomatis* IgG and IgA antibodies in the man were associated with a slightly increased risk for male factor infertility. None of the antibodies in the man were significantly associated with TFI in the woman even though a tendency can be noticed. *C. trachomatis* IgG and IgA antibodies in the man were associated with reduced pregnancy rates (table 4). Interestingly the chances to achieve pregnancy were further reduced if the man was positive in both *C. trachomatis* IgG and IgA. When combining the IgG and IgA results into four different groups (i. e. IgG-/IgA-, IgG+/IgA-, IgG-/IgA+ and IgG+/IgA+) only the IgG+/IgA+ remained a statistically significant predictor of reduced pregnancy rates (paper II, Table 4). The combination of IgG and IgA antibodies apparently increased specificity for detecting an effect on male fertility. Stratification for antibody status in the woman, TFI, or age of the man or the woman (below 35 years, 35 years or older), did not change the results.

The mean follow-up time did not differ between the four groups (3.1, 3.0, 3.0 and 3.2 years respectively). IVF or ICSI treatment did not seem to overcome the negative effects on pregnancy rates associated with *C. trachoma-
Table 3. C. trachomatis serum antibodies in men from infertile couples (n=226), in relation to infertility diagnoses.

<table>
<thead>
<tr>
<th>Diagnoses and C. trachomatis antibodies</th>
<th>No (%) with diagnose with antibody</th>
<th>No (%) without diagnose with antibody</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>17 (24)</td>
<td>27 (17)</td>
<td>1.3</td>
<td>0.82-2.0</td>
</tr>
<tr>
<td>IgA+</td>
<td>12 (17)</td>
<td>16 (10)</td>
<td>1.4</td>
<td>0.87-2.3</td>
</tr>
<tr>
<td>chSP60 IgG+a</td>
<td>14 (21)</td>
<td>27 (19)</td>
<td>1.1</td>
<td>0.67-1.8</td>
</tr>
<tr>
<td>IgG+ / IgA+b</td>
<td>9 (15)</td>
<td>8 (6)</td>
<td>1.7</td>
<td>1.05-2.9</td>
</tr>
<tr>
<td>TFI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>11 (24)</td>
<td>33 (18)</td>
<td>1.3</td>
<td>0.73-2.5</td>
</tr>
<tr>
<td>IgA+</td>
<td>6 (13)</td>
<td>22 (12)</td>
<td>1.1</td>
<td>0.50-2.4</td>
</tr>
<tr>
<td>chSP60 IgG+a</td>
<td>12 (27)</td>
<td>29 (17)</td>
<td>1.5</td>
<td>0.85-2.7</td>
</tr>
<tr>
<td>IgG+ / IgA+b</td>
<td>4 (11)</td>
<td>13 (9)</td>
<td>1.3</td>
<td>0.50-3.2</td>
</tr>
<tr>
<td>Unexplained</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>4 (16)</td>
<td>40 (20)</td>
<td>0.79</td>
<td>0.28-2.2</td>
</tr>
<tr>
<td>IgA+</td>
<td>4 (16)</td>
<td>24 (12)</td>
<td>1.3</td>
<td>0.49-3.7</td>
</tr>
<tr>
<td>chSP60 IgG+a</td>
<td>3 (12)</td>
<td>38 (20)</td>
<td>0.59</td>
<td>0.18-1.9</td>
</tr>
<tr>
<td>IgG+ / IgA+b</td>
<td>3 (13)</td>
<td>14 (8)</td>
<td>1.5</td>
<td>0.49-4.6</td>
</tr>
</tbody>
</table>

a211 men had valid data in chSP60 IgG analysis. 15 men had indeterminant results and were excluded from analysis.
b188 men were either positive in both IgG and IgA or negative in both and included in this analysis.
RR, relative risk (for disease if antibody positive); CI, confidence interval; TFI, tubal factor infertility.

Table 4. C. trachomatis serum antibodies in men from infertile couples (n=226) in relation to pregnancy rate and pregnancy outcome.

<table>
<thead>
<tr>
<th>Pregnancy resultsa and C. trachomatis antibodies</th>
<th>No (%) of pregnant with antibody</th>
<th>No (%) of not pregnant with antibody</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achieved pregnancy</td>
<td>143</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>20 (14)</td>
<td>24 (29)</td>
<td>0.67</td>
<td>0.47-0.95</td>
</tr>
<tr>
<td>IgA+</td>
<td>12 (8)</td>
<td>16 (19)</td>
<td>0.65</td>
<td>0.41-1.005</td>
</tr>
<tr>
<td>chSP60 IgG+b</td>
<td>28 (21)</td>
<td>13 (17)</td>
<td>1.1</td>
<td>0.86-1.4</td>
</tr>
<tr>
<td>IgG+ / IgA+b</td>
<td>4 (3)</td>
<td>13 (19)</td>
<td>0.35</td>
<td>0.14-0.83</td>
</tr>
<tr>
<td>Passed 28 weeksb</td>
<td>109</td>
<td>34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG+</td>
<td>17 (9)</td>
<td>3 (6)</td>
<td>1.1</td>
<td>0.92-1.4</td>
</tr>
<tr>
<td>IgA+</td>
<td>10 (16)</td>
<td>2 (9)</td>
<td>1.1</td>
<td>0.84-1.5</td>
</tr>
<tr>
<td>chSP60 IgG+b</td>
<td>24 (23)</td>
<td>4 (14)</td>
<td>1.1</td>
<td>0.93-1.4</td>
</tr>
<tr>
<td>IgG+ / IgA+b</td>
<td>3 (3)</td>
<td>1 (3)</td>
<td>1.0</td>
<td>0.57-1.9</td>
</tr>
</tbody>
</table>

aAfter 14–54 months (mean 37 months) of follow-up.
b211 men had valid data in chSP60 IgG analysis. 15 men had indeterminant results and were excluded from analysis.
c188 men were either positive in both IgG and IgA or negative in both and included in this analysis.
dNumber of couples that passed 28 weeks of gestation out of the 143 couples that achieved pregnancy.
RR, relative risk (for disease if antibody positive); CI, confidence interval.
tis antibodies (table 5), however the numbers were too small in that group to draw any firm conclusions. The association of *C. trachomatis* antibodies in the man with reduced pregnancy rates is similar to those of some but not to others. In one study, high titers of *C. trachomatis* antibodies in the man were associated with reduced pregnancy rates, however also with TFI in the female partner. The authors concluded that the negative effect was probably based on sexual transmission to the female partner thereby causing damage to the female reproductive tract.

Semen characteristics in relation to *C. trachomatis* antibodies are described in table 6 (for details see paper II, table 5). There were subtle negative changes in semen characteristics associated with *C. trachomatis* IgG and IgA. If both serum *C. trachomatis* IgG and IgA were positive in the man there was reduced sperm concentration and percentage motile spermatozoa, increased teratozoospermia index (TZI), reduced sperm vitality and occurrence of leukocytes in seminal fluid. Whether there is a direct effect on spermatozoa has until recently been controversial. There is accruing evidence of detrimental effects of *C. trachomatis* on semen characteristics. Reduced motility, sperm DNA fragmentation, reduced survival of spermatozoa, lowered sperm concentration, blunt ed acrosome reaction and an increased amount of leukocytes are among the findings. Others have not found any associations between serum or semen IgG or IgA and semen characteristics. In an in vivo study on infertile men (n = 143) the effect of a *C.
trachomatis infection was greatest, in relative terms, on sperm DNA fragmentation as compared to standard semen parameters. A subgroup (n = 95) of patients were evaluated after antibiotic treatment and showed a significant improvement in sperm DNA fragmentation and also in sperm morphology. The follow-up in terms of clinical pregnancies was somewhat irregular and considered only a very small subgroup; out of 14 couples attempting pregnancy 3-6 months after antibiotic therapy, 12 (86%) achieved pregnancy. Sperm DNA fragmentation has in meta-analyses been associated with reduced pregnancy rates and increased risk of pregnancy loss after IVF and ICSI. There was in this study no association of C. trachomatis antibodies in either the man or the woman with pregnancy outcome/loss.

Even though the above mentioned implicates that there might be a direct effect of C. trachomatis on sperm quality, and male fertility, it cannot be ruled out that the effect is a result of a factor(s) that covaries with C. trachomatis serum antibodies and is as yet unknown. One of many plausible factors could be a concurrent undiagnosed infection with M. genitalium, that is also sexually transmitted and is an established cause of non-gonococcal urethritis and is shown to attach to human spermatozoa. The Practice Committee of the American Society for Reproductive Medicine has reviewed available data on cigarette smoke and effect on fertility and conclude that there is reduced sperm concentration, motility and morphology in men that are cigarette smokers, but the effects on pregnancy rates have been difficult to discern. Data on smoking was not available for the women and men in this study, neither was serum cotinine, a nicotine metabolite used to determine the amount of cigarette smoke exposure, analyzed. The decreased pregnancy rates might also, in part, be an effect of sexual transmission to the female partner causing damage to the female reproductive tract. C. trachomatis may also, by causing epididymitis, damage the canalicular system of the male genital tract thereby causing obstructive azoospermia, although this is probably a rare event and cannot account for the results herein. Another theory is that C. trachomatis may induce an autoimmune cross-reaction with antisperm antibodies or autoantibodies based on immune activation. There was no association of serum C. trachomatis antibodies in the man and seminal antisperm antibodies in this study.

Methodological considerations

Strengths of this study were the homogenous cohort of 244 (reduced to 226 in paper II) consecutive couples where both the man and the woman were included in the analysis. There was a long follow-up period with a small number of drop-out. The serum antibody tests used are specific for C. trachomatis serovar D–K known to cause genital disease. However, tubal assessment might have been more accurate if all women had undergone laparoscopy with chromo-perturbation where also peritubal adhesions could have been detected. Laparoscopy was carried out only if hysterosalpingosonography (HSS) did not show patent, un-dilated tubes bilaterally, or if indicated for other reasons like suspicion of endometriosis. This should give a fairly accurate diagnosis of TFI since sensitivity of HSS for tubal occlusion/dilation is shown to be more than 88%. It would also be an ethical dilemma to subject women to laparoscopy if HSS is sufficient. C. trachomatis DNA testing on a first-void urine specimen was performed only if one of the partners of the couple was positive in serum C. trachomatis IgG. Testing all would have resulted in a more reliable estimate of the prevalence in the infertile cohort. Theoretically, though, not many persons would have been positive if negative in IgG testing since there was a significant correlation of C. trachomatis IgG titer levels and the finding of C. trachomatis
DNA \( (r = 0.3, P = 0.026) \). Since bacteria might be present in semen\textsuperscript{231, 232} or prostatic tissue,\textsuperscript{233} even though not evident in urine specimens, it would have been plausible to test also semen samples for \textit{C. trachomatis} DNA. The results of couples treated by insemination should have been divided in two groups; insemination with husband sperm (AIH) or donor sperm (AID), since the treatments are different, analyzing the impact of \textit{C. trachomatis} in the male partner regarding pregnancy rates. Data on cigarette smoking is lacking in both women and men why possible confounding from this factor could not be accounted for. To be able to draw any conclusions on whether the negative effects of \textit{C. trachomatis} can be overcome with IVF/ICSI a larger cohort of IVF/ICSI treated couples is needed.

Different antibodies – different mechanisms?

Serum cHSP60 IgG antibodies are consistently associated with sequelae of chlamydial infection in women, such as PID, TFI, and ectopic pregnancies.\textsuperscript{37-40, 42, 43, 102, 234-239} Chlamydial HSP60 IgG antibodies were also associated with a subgroup of ovarian tumors in the women in paper III, while \textit{C. trachomatis} IgA antibodies were unrelated to any kind of ovarian tumors as well as infertility parameters in the women of the present studies. On the contrary, cHSP60 IgG antibodies were not associated with male infertility either in this study or the study by Karinen et al.\textsuperscript{205} Instead, a stronger association with both reduced pregnancy rates and impaired semen characteristics was found when the man was positive in both serum \textit{C. trachomatis} IgG and IgA antibodies. It is intriguing to speculate how the difference in antibodies translates into different mechanisms of disease. In women tissue damage of the fallopian tubes is the main characteristic of \textit{C. trachomatis} induced infertility, caused by persistent or repeated \textit{C. trachomatis} infections. When \textit{C. trachomatis} enters a persistent state it produces predominantly cHSP60 proteins thereby inducing the production of cHSP60 antibodies. If the tissue damage is caused by the bacteria itself and the inflammatory reaction surrounding it, or by an autoimmune cross-reaction of cHSP60 antibodies to human HSP60 thereby attacking “self” tissue, or both, is not clearly understood.

In the man, serum cHSP60 IgG antibodies were unrelated to pregnancy rates and did not add any extra information regarding semen characteristics. Tissue damage by persistent \textit{C. trachomatis} infections is probably not the main component of the negative effects on fertility. IgA antibodies, that were more informative regarding the effects on male fertility, are more likely to reflect an active on-going phase of the infection as compared to cHSP60 IgG antibodies. A combination of both IgG and IgA antibodies seemed to increase the specificity in detecting decreased fertility potential and impaired semen characteristics. Hence, indicating that it is chronic (or acute) infection with \textit{C. trachomatis}, and bacteria present in the male reproductive tract, exerting its effects directly to the spermatozoa, that impair male fertility. This is supported by in-vitro studies where co-incubation of human sperm with \textit{C. trachomatis} bacteria results in increased phosphatidylserine externalization (a sign of apoptosis) and sperm DNA fragmentation,\textsuperscript{128} and reduced motility and vitality.\textsuperscript{127} In vivo \textit{C. trachomatis} has been associated with sperm DNA fragmentation\textsuperscript{221} as well as blunted acrosome reaction.\textsuperscript{107}

Papers III and IV: Ovarian tumor study

Prevalence of \textit{C. trachomatis} and \textit{M. genitalium} antibodies

The prevalence of plasma antibodies among cases and controls are given in Figure 9A-C, but are also presented in paper III (table 2) including the \( P \) - value of the association between a specific antibody and ovarian tumors. The prevalence of plasma \textit{C. trachomatis} IgG,
Benign conditions, n = 209; BOT, borderline ovarian tumors, n = 12/matched controls n = 48; EOC, epithelial ovarian cancer, n = 47/matched controls n = 188; other malignancies n = 9/matched controls n = 35.
cHSP60 IgG and *M. genitalium* IgG antibodies in women undergoing surgery due to suspected ovarian pathology were generally high. Interestingly, the prevalence of *C. trachomatis* IgG antibodies in the infertile patient group (paper I and II) (mean age 31.1 years) from the same region during 1997-2001, using the same methods, was similar (24%) as in the ovarian tumor group (22%), and the control group (16%) compares to the fertile controls (16%).

Women with ovarian tumors presented cHSP60 IgG antibodies more often than the infertile women (25% vs. 16%). One could speculate if this is due to a high percentage of persistent infections expressing high levels of cHSP60, and long persistence of antibodies accumulating to high prevalence in older age among women with ovarian tumors. Or are infertility and low parity confounders to *C. trachomatis* antibodies since they per se are (suggested) risk factors for ovarian cancer. Alternatively, a past or persistent infection might be part of the pathogenesis in ovarian cancer as well as in infertility.

*C. trachomatis* and *M. genitalium* antibodies in relation to ovarian tumors

*C. trachomatis* antibodies were not significantly associated with ovarian tumors, neither any of the tumor subgroups (BOT, EOC, serous, mucinous, endometrioid, clear cell, mixed or undifferentiated) when plasma samples drawn maximum one year prior to diagnosis were considered. However, cHSP60-1 IgG antibodies were associated with EOC when plasma samples drawn more than one year prior to diagnosis (n = 11) were analyzed (vs. women with benign conditions: OR = 5.6, 95% CI 1.5 – 20; vs. matched controls OR = 4.2, 95% CI 0.97 – 18). The carcinomas were reclassified according to the hypothesis of a type I and a type II pathogenetic pathway, and the type II tumor group was studied further. When plasma samples drawn more than one year prior to diagnosis (n = 7) were analyzed (figure 10), an association of cHSP60-1 IgG antibodies with type II tumors compared with matched controls (OR = 25, 95% CI 2.4 – 260) and women with benign conditions (OR = 19, 95% CI 2.2 – 164) was found. The prevalence of cHSP60-1 IgG antibodies was 86% in plasma samples drawn more than one year prior to diagnosis, compared to 16% in plasma samples drawn a few days prior to diagnosis, in the type II tumor group. This indicates that there must have been a rapid loss of antibodies during the year(s) before diagnosis, possibly after tumor inception. Persistence of cHSP60 IgG antibodies is not previously stud-
ied but *C. trachomatis* IgG antibodies are in some studies shown to decrease in a high percentage of cases\textsuperscript{242, 243} but not in others.\textsuperscript{241, 244}

Since the type II tumor group with prospective plasma samples was small, concern is raised that the high prevalence is due to chance alone. Furthermore, the possibility to correct for potential confounders was limited. However, data on clinical characteristics in the subgroup of type II tumors was well-documented and the group was not extreme in any other respect than the prevalence of chHSP60-1 IgG antibodies (paper III, table 4). A rule of thumb is that a multiple logistic regression model needs at least 10 cases for each variable to be included in the analysis, and a minimum requirement is twice as many subjects as independent variables.\textsuperscript{245} None of the potential confounders (age at menarche, age at menopause, parity, oral contraceptive pill use, HRT use, smoking and BMI) changed the significance of the association between chHSP60-1 IgG antibodies and type II tumors in a multivariate analysis. Due to the small number of cases precision of the estimate of the association is poor and no conclusion on the strength of the relative risk associated with chHSP60-1 IgG antibodies can be inferred from this data. Nonetheless, a significant association of chHSP60-1 IgG antibodies with type II ovarian/pelvic cancer was present.

Furthermore, plasma *M. genitalium* IgG was associated with BOT (n = 12) (vs. matched controls: OR = 11, 95% CI 1.7 – 74; vs. benign conditions: OR = 3.8, 95% CI 1.09 – 13). *C. trachomatis* IgG and chHSP60-1 IgG antibodies were only slightly raised in the BOT subgroup. This subgroup was also small leading to poor precision in the estimates. The association of *M. genitalium* IgG antibodies with BOT might represent a type 1 error since a correction for multiple comparisons according to Bonferroni-Holm,\textsuperscript{203} defining the tests performed on BOT, EOC and other pelvic malignancies (paper III, table 2) as one family, eliminates the significance of the results. Possible mechanisms by which *Mycoplasma* might act as a tumor initiator/promoter (besides chronic inflammatory changes) are not known, but the Mycoplasma membrane lipoprotein p37 of *M. hyorhinis* is suggested to promote malignant changes in mammalian cells.\textsuperscript{246–248} The gene expression profile is altered during Mycoplasma-induced malignant cell transformation\textsuperscript{249} and *Mycoplasma* causes inhibition of p53 tumor suppression function and activation of nuclear factor κB (NF-κB) in rodent fibroblasts.\textsuperscript{250} If this applies to *M. Genitalium* and BOT remains unknown.

*C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, HPV, and polyoma virus BKV and JCV in ovarian tissues (paper IV).

All 312 ovarian tissue samples analyzed were positive for the human β-globin gene and considered to have sufficient DNA quality. None of the tissue samples were positive for *M. genitalium*, HPV, JCV or BKV DNA.

Urine samples positive or negative for *C. trachomatis* with the Becton Dickinson ProbeTec PCR method were confirmed positive and negative as expected with the Aptima Combo 2 test. Likewise the ovarian tissue biopsies spiked with *C. trachomatis* bacteria were all, after RNA-extraction, transportation and handling as with the test tissues, confirmed positive. None of the 312 ovarian tissue samples were positive for *C. trachomatis* or *N. gonorrhoeae* rRNA.

Methodological considerations

The study population represented all cases in a well-defined geographic area during a specified time period. However, plasma and tissue samples were lacking in a considerable number of cases (Figure 7). Women without plasma or tissue samples did not differ in terms of age or diagnoses from the women with plasma or tissue eligible for analysis though. Furthermore, data on general, gynecological.
cologic and reproductive health were not complete, particularly among women with benign conditions, since they were extracted from the medical records. Plasma samples drawn within a few days prior to diagnosis were analyzed in a majority of cases. Considering the results of this study, and others concerning *C. trachomatis* and cervical cancer and *H. pylori* and gastric cancer (will be discussed in *Suggested model of C. trachomatis in the pathogenesis of ovarian cancer*), analyzing plasma samples drawn several years prior to diagnosis would have been a more plausible approach.

We chose to compare the antibody prevalence among the women with BOT, EOC and other malignancies with the prevalence among women with benign conditions. However, women going through surgery due to benign conditions might have bleeding irregularities, pain, infertility, etc. Sexually transmitted infections, especially *C. trachomatis*, are known to be a possible cause of many of these conditions, rendering the possibility of a high prevalence of antibodies in this patient group. Using them as controls might have lead to an underestimation of the possible association of *C. trachomatis* and *M. genitalium* antibodies with ovarian tumors. The women with benign conditions were also younger than the women with carcinomas. Therefore, an external control group of women without a cancer diagnosis and alive at the time of the diagnosis of the index case was also analyzed. The control plasma samples were collected as part of the Northern Sweden Health and Disease Cohort (NSHDC) which is a population based, prospective health survey cohort, in Västerbotten County, serving the same geographic area as the cases were recruited from. Four controls per case were matched with respect to age and date of plasma sampling. This is important as the prevalence of sexually transmitted infections (STI’s) is known to vary over time and the incidence and prevalence are different in different age groups. The participation rate of the Västerbotten Intervention Project (VIP), the part of NSHDC from which a vast majority of the controls were recruited, has varied between 55% and 60% of all the eligible persons invited during the course of the program. The participants have been compared with the non-participants and no obvious social differences have been found. By using two control populations we attempted to increase the basis for the interpretation of data. There was a difference in *C. trachomatis* IgG antibody prevalence among women with benign conditions compared with controls from the NSHDC (24% vs. 16%, OR 1.7, 95% CI 1.08 – 2.7). No differences in chHSP60-1 IgG or *M. genitalium* IgG antibodies were detected.

The prevalence of chHSP60-1 IgG antibodies in this study was relatively high compared with the prevalence of *C. trachomatis* IgG antibodies, particularly in the subgroup of women with type II carcinoma. This is different from the findings in most other studies and might reflect differences in laboratory methods. It also raises the question if there was a high number of false positive tests, i.e. poor specificity. Another explanation is that the *C. trachomatis* infections that will eventually lead to carcinomatous changes produce higher amounts of chHSP60 or produces chHSP60 for a more protracted period of time, e.g. persistent infections for a long period of time, inducing the production of chHSP60 IgG antibodies. Alternatively, there is a difference in the host immune system that puts *C. trachomatis* bacteria in a persistent state, leading both to the continuous production of chHSP60 IgG antibodies and renders the host more susceptible to carcinomatous changes. This might be the case if for example an autoimmune cross-reaction to the human HSP60 plays a pathological role. Differences in host immunological traits have been associated with development of PID, TFI, recurrent infections, and tubal pathology.
Since none of the microorganisms analyzed were found in any of the tissue samples one is reluctant to think that the methods used were insufficient. The nucleic acid extraction methods used are well-documented commercial kits, and the presence of human DNA was tested in all extractions. DNA quality, though, was not examined by targeting a human RNA gene in the tissue samples. However, RNA integrity in snap-frozen tissues stored at -80°C subjected to repeated freeze-thaw procedures is shown to be intact. The extraction, transportation and analysis of RNA were validated both by running positive C. trachomatis urine samples and C. trachomatis spiked ovarian biopsies through the same process with the expected results. Nonetheless, concern might be raised if RNA was degraded in these tissue samples, or if targeting rRNA is the right method for detecting a persistent C. trachomatis infection. However, rRNA is shown to be continuously expressed in experimentally induced persistent infections. Analyzing the presence of human RNA in the tissue samples or using a NAAT method targeting C. trachomatis and N. gonorrhoeae DNA could have helped solve the question.

M. genitalium, HPV and polyoma virus were analyzed with NAATs using DNA as target and with a target length shorter than the human β-globin gene used as a positive extraction control. The NAATs used are well-documented. All together this indicates that there is a true absence of M. genitalium, HPV or polyoma virus in the ovarian tissue samples.

Does the absence of bacteria and virus in the ovarian tissue mean that they are not part of the pathogenesis in ovarian tumor development? Wallin et al. found all tumor biopsies from cervical cancer at diagnosis to be negative for C. trachomatis. However, C. trachomatis DNA in cervical pap smears several years prior to the diagnosis was associated with cervical cancer. Furthermore, the serological association is stronger the longer the lag time is between blood sampling and cervical cancer diagnosis. Concerning gastric cancer the association between H. pylori infection and cancer is strongest when the interval between serum collection and cancer diagnosis is longer than 15 years. When atrophic gastritis and intestinal metaplasia occur (precursors of gastric adenocarcinoma), biopsies taken from these areas yields no H. pylori bacteria. However, H. pylori is often identified in nonatrophic locations of the same stomach where intestinal metaplasia occurs, but C. trachomatis bacteria could not be detected in any ovarian tissues (malignant, benign or from the contralateral ovary) in this study. No tissue from the fallopian tubes or the tubal fimbriae were analyzed which might have been a more plausible approach considering the hypothesis that type II ovarian/pelvic cancer arises in the tubal fimbriae, with a rapid spread to the ovaries soon after inception.

Suggested model of C. trachomatis in the pathogenesis of ovarian cancer

The results in this study will be put into the context of the present knowledge of C. trachomatis infections and ovarian tumor pathogenesis, suggesting a hypothesis for an association by trying to answer three questions. When, where and how could possibly a C. trachomatis infection contribute to ovarian carcinogenesis?

When does C. trachomatis initiate/promote ovarian carcinogenesis?

Chlamydial HSP60-1 IgG antibodies were associated with ovarian cancer only when plasma samples drawn more than one year prior to diagnosis were analyzed, and the bacteria were not present in ovarian tumors at diagnosis. Similar to this, the association of H. pylori infection with gastric cancer (which are etiologically linked) is stronger the longer the interval between serum collection and cancer
diagnosis, and \textit{H. pylori} bacteria are not found in gastric adenocarcinoma tissue.\cite{251}

The results herein have similarities also with the findings concerning \textit{C. trachomatis} and cervical cancer. Naucler et al. observed a positive correlation between \textit{C. trachomatis} IgG antibodies and cases observed during follow-up, but not with cases identified at baseline;\cite{263} IgG antibodies have been associated with cervical cancer in cases with lag time more than 3.5 years between serum sampling and cancer diagnosis;\cite{45} an increasing risk has been associated with increased lag time\cite{45} and if the lag time was more than 12 months there was an association between antibodies and cervical carcinoma.\cite{146}

Furthermore, prediagnostic smears and cervical cancer tissue were devoid of \textit{C. trachomatis} even though the bacteria were present in Pap smears several years before cancer diagnosis.\cite{262} No \textit{C. trachomatis} DNA was identified in Pap smears from control women.

\textit{C. trachomatis} antibodies were not associated with ovarian cancer in a study where retrospectively drawn plasma samples were analyzed\cite{154} but an association of borderline-significance was found in another study.\cite{153} A conceivable explanation for this is that antibodies might decline over time\cite{242,243} and new infections occur that will dilute a possible association. Alternatively, the ovarian cancer treatment influences \textit{C. trachomatis} antibody prevalence.

\textbf{Where is the site for ovarian carcinogenic \textit{C. trachomatis} infections?}

\textit{C. trachomatis} is known to cause damage to the distal fallopian tube but the bacteria have been detected in many cell types of the human body. However, in experimentally induced infections of mice\cite{264} as well as pig-tailed monkeys,\cite{265} where the histopathologic changes were characteristic of the damage observed in women with TFI,\cite{266} chlamydial inclusions were observed only in the secretory cells both by immunoperoxidase staining and by transmission electron microscopy. \textit{C. trachomatis} bacteria have not been identified in the ovarian tissue in this study. Are the secretory cells the main target of \textit{C. trachomatis} in the fallopian tubes? If so, it coincides with the target of the p53 signatures that are associated with tubal intraepithelial carcinoma (TIC),\cite{142} the suggested precursor of type II ovarian/pelvic carcinomas.\cite{267} The mucosa of the tubal fimbriae have shown to be resistant to implantation from carcinomas at other sites of the abdomen, whereas the ovarian surface is a well-known target for metastases from the gastro-intestinal region.\cite{268} By using protocols for the examination of the tubal fimbriae that maximize the exposure of the mucosa in serous ovarian, primary peritoneal and tubal carcinomas, foci of coexisting TIC were identified in 20 of 30, 4 of 6 and 5 of 5 carcinomas respectively.\cite{267} In the same study, the p53 mutations of the TIC and serous ovarian cancer were analyzed in five cases, and found to be identical in the coexisting TIC and the ovarian tumor mass in all five. This supports the possibility that the carcinomas considered ovarian in origin actually have its site for carcinogenesis in the secretory cells of the tubal fimbriae, known to be damaged by \textit{C. trachomatis} infections.

\textbf{How does the \textit{C. trachomatis} infection initiate/promote malignant transformation?}

Persistence of \textit{C. trachomatis} infections are known to have an altered steady-state level of chlamydial antigens, with the predominance of cHSP60 protein compared to the level of the major outer membrane protein (MOMP).\cite{13,33,260} This is a feature believed to be part of the survival strategy for the bacteria since cHSP60 is suggested to have anti-apoptotic properties.\cite{35} It is of vital importance to the \textit{C. trachomatis} bacteria that the host cell does not undergo apoptotic changes due to the obligate intracellular developmental cycle. At the
same time the inflammatory reaction causes production of toxic oxidants, cytokines, prostaglandins and growth factors that might cause damage to the DNA. Increased cell-turnover is also part of the inflammatory reaction with potential for replication errors and mutations, while cHSP60, by having an anti-apoptotic effect, facilitates the survival of DNA-damaged cells leading to increased risk for malignant transformation.46

Chlamydial HSP60-1 IgG was associated with type II carcinomas in this study when prospective plasma samples were analyzed, indicating that the carcinomas might have been preceded by a chlamydial infection dominated by cHSP60 production. Since cHSP60 is suggested to have anti-apoptotic properties and type II tumors are characterized by a high level of \( p53 \) mutations (a marker of apoptosis related genetic instability), cHSP60 might act as a tumor promoter by facilitating the survival of cells with \( p53 \) mutations. In 16 of the BOT and EOC cases in this study where cHSP60-1 IgG antibodies were analyzed, the extent of \( p53 \) mutations had previously been semi-quantitatively (0, 1, 2, 3) determined by immunohistochemistry.269 Interestingly, the level of \( p53 \) staining was associated with the presence of cHSP60-1 IgG antibodies (\( P = 0.002 \), Mantel-Haenszel test).

Even though in this study the number of cases showing an association of \( C. trachomatis \) antibodies and type II ovarian cancer was small, and need to be confirmed in larger studies, the answers to the when, where and how questions could be summarized in the following hypothesis: \( C. trachomatis \) might initiate/promote ovarian cancer development by persistently infecting the secretory cells of the tubal fimbriae many years, or decades, before the tumor is overt. The tumor promoting/initiating effect can be caused by \( C. trachomatis \) bacteria inducing chronic inflammation, directly and/or by an auto-immune cross-reaction to human HSP60, with the production of cytotoxic substances. Furthermore, cHSP60 mediated inhibition of apoptosis of DNA-damaged cells can lead to malignant transformation of the secretory cells of the tubal fimbriae, followed by rapid spread to the ovaries and the peritoneal lining in close proximity. Soon, there is manifest epithelial ovarian carcinoma.
Summary and Conclusions

*Chlamydia trachomatis* is suggested to have a broad spectrum of effects in the human body. To facilitate an intracellular developmental cycle *C. trachomatis* has developed tools to modify the regulatory mechanisms of the host cell, at the same time causing effects that are unwanted by the host. In this thesis, data on possible effects on female fertility, male fertility and ovarian tumor development is presented.

*C. trachomatis* IgG and IgA antibodies in combination in the man were independently associated with substantially reduced pregnancy rates for the infertile couple, and subtle negative effects on semen characteristics. Lately, papers supporting a direct negative effect by *C. trachomatis* on male fertility, and spermatozoa, are accumulating. Regarding female fertility, *C. trachomatis* is known to have negative effects, increasing the risk for TFI, which was supported by data in this study. A tendency, however not significant, to reduced pregnancy rates after IVF-treatment if the woman was *C. trachomatis* IgG antibody positive was also found.

It has been hypothesized that *C. trachomatis* might increase the risk for ovarian cancer. However, it is not previously studied whether *C. trachomatis* antibodies are associated with the recently hypothesized type II tumor subgroup. In this thesis a novel finding of an association of cHSP60-1 IgG antibodies with type II ovarian/pelvic carcinoma is presented along with a suggested model of how *C. trachomatis* might act as a tumor initiator/promoter. Since the subgroup of type II tumors was small, the results have to be confirmed in future studies.

The principal findings of the present thesis can be itemized as follows:

- *C. trachomatis* seems to exert a direct effect on male fertility. Serum *C. trachomatis* IgG and IgA antibodies in the man were associated with reduced pregnancy rates for the infertile couple, as well as subtle negative changes in semen characteristics.
- In the woman *C. trachomatis* infections, as detected by serum *C. trachomatis* IgG and cHSP60 IgG antibodies, increased the risk for TFI, but did not significantly reduce pregnancy rates in this cohort of infertile couples.
- Pregnancy outcome, up to 28 weeks of gestation, did not differ between couples with serum *C. trachomatis* antibodies in either of the partners, and couples without *C. trachomatis* antibodies.
- Type II ovarian/pelvic carcinomas were associated with cHSP60-1 IgG antibodies in women with plasma samples obtained more than one year prior to diagnosis.
- Using TMA, *C. trachomatis* rRNA could not be detected in ovarian tissues from women with epithelial ovarian cancer, borderline ovarian tumors or benign conditions.
• *M. genitalium* plasma antibodies were associated with BOT; however a statistical type 1 error cannot be excluded. *M. genitalium, N. gonorrhoeae*, HPV and polyomaviruses BKV and JCV could not be detected in ovarian tissues.
Suggestions for future research

The finding, in this study, of impaired fertility of the man if he is *C. trachomatis* IgG and IgA positive has to be confirmed in other settings. To further elucidate how *C. trachomatis* affects male fertility, IVF and ICSI treated couples could be studied regarding *C. trachomatis* antibody status, semen characteristics, sperm DNA fragmentation, fertilization rates, embryo development and pregnancy rates. A randomized controlled trial, evaluating if antibiotic treatment can increase pregnancy rates in couples where the man is *C. trachomatis* IgG and IgA positive, would be of great value.

Do *C. trachomatis* infections have an effect on female infertility beyond causing tubal damage? Larger samples of IVF and ICSI treated couples with detailed information regarding *C. trachomatis* IgG and cHSP60 IgG antibodies as well as tubal status, fertilization rate, embryo development and pregnancy rates could help solve this question. It is important in future studies that both partners are evaluated regarding *C. trachomatis* antibodies to avoid confounding of the effects between the partners.

*M. genitalium* is another microorganism suggested to cause TFI in women and urethritis in men. The effect on fertility, and possible confounding, of this microorganism in both women and men should be further evaluated.

Studies using plasma samples obtained more than one year prior to ovarian cancer diagnosis, focusing type II tumors in relation to cHSP60-1 IgG and *C. trachomatis* IgG antibodies, and with sufficient data on possible confounding factors, have to be carried out to verify or reject the results of the present study. Furthermore a larger number of women with BOT should be studied to confirm or reject a possible role of *M. genitalium* in the development of BOT.

If the association of cHSP60-1 IgG antibodies and type II tumors can be confirmed, the next step could be to carefully examine tubal fimbriae mucosa concerning the presence of *C. trachomatis* antigen, cHSP60 proteins and the suggested type II tumor precursors (p53 signatures and TIC), preferably before ovarian cancer is established. This could be done in women who undergo prophylactic salpingo-oophorectomy due to hereditary increased ovarian cancer risk, or in women who undergo salpingectomy due to cervical or endometrial cancer, or for benign conditions.

Research on a vaccine directed towards *C. trachomatis* is accumulating. If *C. trachomatis* in the future becomes an established risk factor for male infertility and ovarian cancer, in addition to female reproductive sequelae, the impetus for vaccine development will increase greatly.
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