Liisa Karinen

CHRONIC CHLAMYDIAL INFECTION: IMPACT ON HUMAN REPRODUCTIVE HEALTH

REPRODUCTIVE HEALTH RESEARCH IN THE NORTHERN FINLAND 1966 BIRTH COHORT (NFBC1966)
LIISA KARINEN

CHRONIC CHLAMYDIAL INFECTION: IMPACT ON HUMAN REPRODUCTIVE HEALTH
Reproductive health research in the Northern Finland 1966 Birth Cohort (NFBC 1966)

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium 4 of Oulu University Hospital, on March 31st, 2006, at 12 noon

OUULUN YLIOPISTO, OULU 2006
Abstract

Chlamydiae are obligatory intracellular gram-negative bacteria with a unique growth cycle. They are very successful pathogens and responsible for a wide variety of infections in humans and different animal species. In addition, they have a tendency to cause recurrent, persistent or chronic infections with potentially severe sequelae years or decades later.

The general purpose of this work was to study the possible serological associations between chronic chlamydial infection, systemic inflammation and reproductive health in a general population. The chlamydial heat shock proteins 60 and 10 (Hs10 and Hsp60) have been suggested to contribute to the pathogenesis of chronic chlamydial infections. Thus, the antibodies to chlamydial Hsp10 and Hsp60 were also investigated in complications of pregnancy.

The present study was a longitudinal population-based birth cohort study, and all of the original papers of this dissertation are based on a nested case-control design.

Our results confirmed the serological association between Chlamydia trachomatis infections and subfertility and the rather high incidence of undiagnosed Chlamydia trachomatis infections in the male partners of subfertile couples. We further demonstrated a serological association between previous Chlamydia trachomatis infections, immunity to chlamydial Hsps and female subfertility. We also showed that serological markers of chronic chlamydial infection present as early as the first trimester are associated with preterm delivery among nulliparous women. When elevated levels of Chlamydia trachomatis IgG and hsCRP were present, the estimated risk for preterm delivery was over 4-fold.

According to our study, nulliparous women who subsequently developed preeclampsia leading to preterm delivery, which was used as a marker of more serious illness, had significantly more often serum IgG antibodies to Chlamydia pneumoniae during the first trimester of pregnancy compared to the preeclamptic women who delivered at term.

In conclusion, chronic Chlamydia trachomatis infection was found to associate with subfertility both in men and in women. In addition, a subclinical chronic inflammatory process associated at least partly with chronic Chlamydia trachomatis infection and present in the first trimester already may be important in the development of preterm delivery. Chronic Chlamydia pneumoniae infection and systemic low-grade inflammation were found to associate with pregnancies that lead to preeclampsia and preterm delivery.

Keywords: chlamydial antibodies, CRP, gestational hypertension, preeclampsia, preterm birth, subfertility
To my family
with love, as always
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Oulu, February 2006

Liisa Karinen
**Abbreviations**

bp  Base pair  
BV  Bacterial vaginosis  
CAT  Chlamydia antibody testing  
CF  Complement fixation  
CI  Confidence interval  
CHsp  Chlamydial heat shock protein  
CRP  C-reactive protein  
DFA  Direct fluorescence assay  
DNA  Deoxyribonucleic acid  
EB  Elementary body  
EIA  Enzyme immunoassay  
FMBR  Finnish Medical Birth Register  
FMC  Finnish Maternity Cohort  
GH  Gestational hypertension  
hHsp  Human heat shock protein  
hsCRP  High-sensitive C-reactive protein  
Hsp  Heat shock protein  
HDR  Hospital Discharge Register  
IFN-γ  Interferon-gamma  
Ig  Immunoglobulin  
Inc  Inclusion membrane protein  
IL  Interleukin  
LCR  Ligase chain reaction  
LGV  Lymphogranuloma venereum  
LPS  Lipopolysaccharide  
MIF  Microimmunofluorescence  
MOMP  Major outer membrane protein  
NGU  Non-gonococcal urethritis  
NFBC  Northern Finland Birth Cohort  
Omp  Outer membrane protein  
Omp2  60 kDa cysteine-rich outer membrane protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Omp3</td>
<td>12 kDa outer membrane protein</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCE</td>
<td>Plasma cell endometritis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>PID</td>
<td>Pelvic inflammatory disease</td>
</tr>
<tr>
<td>Pmp</td>
<td>Polymorphic outer membrane protein</td>
</tr>
<tr>
<td>PROM</td>
<td>Premature rupture of membranes</td>
</tr>
<tr>
<td>PTD</td>
<td>Preterm delivery</td>
</tr>
<tr>
<td>PTL</td>
<td>Preterm labour</td>
</tr>
<tr>
<td>RB</td>
<td>Reticulate body</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operator characteristic</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>SDA</td>
<td>Strand displacement amplification assay</td>
</tr>
<tr>
<td>STD</td>
<td>Sexually transmitted disease</td>
</tr>
<tr>
<td>TFI</td>
<td>Tubal factor infertility</td>
</tr>
<tr>
<td>TMA</td>
<td>Transcription-mediated amplification assay</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TTP</td>
<td>Time to pregnancy</td>
</tr>
<tr>
<td>TWAR</td>
<td>Acronym for <em>Chlamydia pneumoniae</em></td>
</tr>
</tbody>
</table>
List of original publications

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


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1 Introduction

*Chlamydiae* are obligatory intracellular gram-negative bacteria with a unique growth cycle. They are responsible for a wide variety of infections in humans and different animal species. In addition, they have a tendency to cause recurrent, persistent or chronic infections with potentially severe sequelae years or decades later.

*Chlamydia trachomatis* is the most common bacterial cause of sexually transmitted infections throughout the world with high morbidity. *C. trachomatis* can persist in the genital tract for a long period (Dean *et al.* 2000, Witkin 2002), and the diagnosis of chronic infections is problematic. The persistence of genital chlamydial infection has recently been demonstrated in 80.6% of untreated infected men and in 77.6% of untreated infected women (Joyner *et al.* 2002). Several studies have demonstrated an association between *C. trachomatis* seropositivity and tubal factor infertility (TFI) (Punnonen *et al.* 1979, Mol *et al.* 1997). In women, *C. trachomatis* antibody testing has been suggested as part of the routine screening method in infertility investigations (Land & Evers 2002). *C. trachomatis* heat shock proteins 60 (Hsp60) and 10 (Hsp10) have been suggested to participate in the development of female infertility, because antibodies to these proteins are more common in infertile women than in fertile controls (Toye *et al.* 1993, Claman *et al.* 1997, Eckert *et al.* 1997, LaVerda *et al.* 2000, Neuer *et al.* 2000). The value of chlamydial Hsp60 and Hsp10 antibodies in the diagnosis of *C. trachomatis*-associated infertility is, however, under debate (Witkin 2002).

In males, evidence of the link between *C. trachomatis* and infertility is more limited, and there is some controversy concerning the role of both symptomatic and asymptomatic *C. trachomatis* infections in the aetiology of male infertility (Eggert-Kruse *et al.* 1990, Paavonen & Eggert-Kruse 1999, Idahl *et al.* 2004).

Chlamydial infection during pregnancy is associated with a number of adverse outcomes of pregnancy, including preterm delivery, premature rupture of membranes, low birth weight, neonatal death and postpartum endometritis (Andrews *et al.* 2000, Mårdh 2002). Preterm birth is one of the biggest problems in modern obstetrics, and the leading cause of neonatal mortality and morbidity. In pregnant women, acute *C. trachomatis* infection associates with premature rupture of membranes, chorioamnionitis, premature delivery and puerperal and neonatal infections, but the evidence is still mainly indirect (Yost & Cox 2000, Challis 2000). It has been hypothesized that inflammation of the
decidual tissue or chorioamnion leads to prostaglandin production, cervical ripening and subsequent uterine contractions (Romero 1988). It is not known exactly when this inflammatory process begins, or how long a latency period is required before the onset of symptoms. A pre-existing subclinical intrauterine inflammatory process in early gestation has been proposed as a possible antecedent leading to preterm delivery (Wenström et al. 1996, Wenström et al. 1998, Hvilsom et al. 2002). We need to expand our knowledge of the role of chronic *C. trachomatis* infection in the pathogenesis of preterm delivery.

Preeclampsia is a multisystem disorder of unknown causality and unique to human pregnancy. The clinical findings of preeclampsia include elevation of blood pressure and consistent proteinuria of 300 mg/day or more after the 20th gestational week in a previously normotensive woman (Brown et al. 2001). It is characterized by an abnormal vascular response to placentation, which is associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system and endothelial cell dysfunction (Roberts 1998, Redman et al. 1999, Report of the National High Blood Pressure Education Program 2000). Although the etiology of endothelial dysfunction in preeclampsia is unknown, it has been postulated to be part of an exaggerated maternal inflammatory response to pregnancy (Sacks et al. 1998, Redman et al. 1999, von Dadelszen et al. 2003).

*Chlamydia pneumoniae* is a common respiratory pathogen worldwide. It does not only cause acute respiratory infection, but is also associated with chronic pulmonary diseases and coronary heart disease (Leinonen & Saikku 2002). *C. pneumoniae* is invasive: it can multiply in vascular endothelial and smooth muscle cells, which makes its systemic dissemination through circulation possible (Gaydos 1996). Many risk factors and pathophysiological abnormalities of preeclampsia are similar to those of coronary artery disease. The current concept of atherosclerosis as an inflammatory disease may be compatible with the recent evidence suggesting that preeclampsia is an inflammatory condition as well (Teran et al. 2001, Heine et al. 2003, von Dadelszen et al. 2003). On the other hand, preeclampsia and gestational hypertension (*de novo* hypertension during pregnancy without proteinuria) are associated with metabolic syndrome (Pouta et al. 2004). We need to find out more about the role of chronic *C. pneumoniae* infection in the pathogenesis of preeclampsia and gestational hypertension.

In the present population-based study, the role of chronic chlamydial infection in human reproductive health was evaluated by measuring serum *C. trachomatis* and *C. pneumoniae* antibodies, antibodies to the *C. trachomatis* heat shock proteins 60 and 10 (Hsp60 and Hsp10) and C-reactive protein levels by highly sensitive assay (hsCRP) in subjects with subfertility, premature birth and preeclampsia.

This unique study population of males and females from the Northern Finland Birth Cohort (NFBC 1966) surveyed at the age of 31 years provided us with the possibility to evaluate the role of *C. trachomatis* infection in subfertility in a general population. In addition, this population-based birth cohort will provide an opportunity to study the role of chronic *C. trachomatis* and *C. pneumoniae* infections in early pregnancy and the influence of low-grade systemic inflammation during pregnancies and pregnancy complications, especially preeclampsia and preterm delivery, in a population-based setting.
2 Review of the literature

2.1 General aspects of Chlamydiae

2.1.1 Taxonomy

Chlamydiae are obligate intracellular gram-negative eubacteria with a unique biphasic life cycle (Grayston & Wang 1975). Originally, they were taxonomically categorised as a separate order, Chlamydiales, which contained the single family, Chlamydiaceae, and only one genus, Chlamydia (Moulder et al. 1984). The genus included four species, which differ in their host cell tropism, but have similar cell structure and share certain biological properties in the course of their intracellular existence: C. trachomatis, C. psittaci (Moulder et al. 1984), C. pneumoniae (Grayston et al. 1989) and C. pecorum (Fukushi & Hirai 1992).

In 1999, a radical change of the chlamydial taxonomy was proposed on the basis of molecular markers. According to the proposed taxonomy, the family Chlamydiaceae was divided into two genera, Chlamydia and Chlamydophila, with nine species (Everett et al. 1999). However, the proposal to change the taxonomic nomenclature for the Chlamydiaceae family has not been generally accepted in the field (Schachter et al. 2001).

C. trachomatis and C. pneumoniae are the two chlamydial species pathogenic to humans, whereas the other species occur mainly in animals. C. trachomatis has been isolated only from humans and comprises two human biovars, trachoma and lymphogranuloma venereum (LGV), with 21 sero- or genotypes (including subtypes): A, B, Ba, C, D, Da, E, F, G, Ga, H, I, Ia, J, Ja, Jv, K, L1, L2, L2a, and L3 (Wang et al. 1985, Frost et al. 1991, Wang & Grayston 1991, Morré et al. 1998, Dean et al. 2000) on the basis of differences in the Omp 1 gene that encodes major outer membrane protein (MOMP). C. pneumoniae has only one serovar, with almost 100% DNA homology between the strains but less than 10% homology with the other Chlamydiae (Kuo et al. 1995).

C. psittaci is genetically more heterogeneous than C. trachomatis, and the number of serovars is unknown (Everett et al. 1999). C. pecorum, which causes infection in ruminants, was established as a species distinct from C. psittaci in 1992 (Fukushi & Hirai 1992).
The essential features of the three chlamydial species are presented in Table 1.

**Table 1. Essential features of chlamydial species.**

<table>
<thead>
<tr>
<th>Feature</th>
<th><em>C. pneumoniae</em></th>
<th><em>C. trachomatis</em></th>
<th><em>C. psittaci</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural hosts</td>
<td>humans, (koala, horse, frog)</td>
<td>humans</td>
<td>birds and mammals</td>
</tr>
<tr>
<td>Host cell tropism</td>
<td>epithelial cells, mononuclear lymphocytes, endothelial cells</td>
<td>epithelial cells, mononuclear lymphocytes (LGV)</td>
<td>mononuclear lymphocytes, epithelial cells, other cell types</td>
</tr>
<tr>
<td>Major human disease</td>
<td>pneumonia, respiratory tract infection</td>
<td>trachoma, STD</td>
<td>pneumonia abortion</td>
</tr>
<tr>
<td>Transmission</td>
<td>aerosol</td>
<td>sexual, neonatal (STD); hand to eye, flies (trachoma)</td>
<td>aerosol, excretions</td>
</tr>
<tr>
<td>Number of serovars</td>
<td>1(?)</td>
<td>18</td>
<td>unknown</td>
</tr>
<tr>
<td>DNA homology with <em>C. pneumoniae</em> (%)</td>
<td>94–100</td>
<td>&lt;5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>MOMP-containing species-specific antigens</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Sensitivity to macrolides and tetracycline</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Sensitivity to sulphas</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Glycogen in inclusions</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Morphology of elementary body</td>
<td>Pear-shaped or round</td>
<td>round</td>
<td>round</td>
</tr>
</tbody>
</table>

STD=sexually transmitted disease, MOMP= major outer membrane protein
(Source: Schachter 1989, Kuo et al. 1995)

### 2.1.2 Biology of chlamydia

#### 2.1.2.1 Growth cycle

Chlamydiae are intracellular bacteria that have a unique biphasic developmental cycle with two distinct morphological forms. The extracellular, infectious form (0.3 µm) is called elementary body (EB), and the intracellular, replicating form (1.0 µm) is called reticulate body (RB). Infectious EBs start the cycle by attaching to a susceptible host cell membrane. They gain access into the host cell via either parasite-specified phagocytosis or receptor-mediated endocytosis. When inside the cell, the chlamydiae remain within an enlarging intracellular vacuole, a characteristic inclusion, avoiding lysosomal fusion and obviously hence destruction. During the first few hours, EBs differentiate into metabolically active RBs. By using the host cell’s energy and nutrient resources, RBs begin to multiply by binary fission. After multiple rounds of division, RBs start to transform back to EBs. Finally, by exocytosis or host cell lysis, the infectious EBs are released into the
cytoplasm, to initiate new cycles in new host cells. (Reviewed by Abdelrahman & Bel-land 2005.)

In cell culture conditions, the duration of the developmental cycle is between 2 and 3 days. In natural infections, the situation is more complicated, and the normal chlamydia development is easily disturbed. Certain circumstances (nutrient deficiency, interferon-gamma, antibiotics) may result in morphological alterations of RBs and the emergence of enlarged, atypical chlamydial forms (Beatty et al. 1993). These aberrant forms may persist inside the host cell in a viable but nonculturable state for a long time and result in persistent infection despite activation of the immune defence mechanisms. The cycle of both normal and altered development of Chlamydia is presented in Fig. 1.

![Developmental cycle of Chlamydia](image)

**Fig. 1. Developmental cycle of Chlamydia (modified after: Beatty et al. 1994a).**

### 2.1.2.2 Structural characteristics

Similarly to all gram-negative bacteria, chlamydial cells appear to be surrounded by a double membrane. However, unlike the other gram-negative bacteria, Chlamydiae do not have a peptidoglycan layer in the space between the two membranes (Barbour et al. 1982, Fox et al. 1990), although the genes for peptidoglycan synthesis are present in the chlamydial genome (Chopra et al. 1998). The outer cell membrane consists of lipopolysaccharide (LPS) and outer membrane proteins (Omp), the single predominant protein being the MOMP of 38 to 42 kDa, comprising about 60% of the Omps (Caldwell et al. 1981). The MOMP of *C. trachomatis* contains serovar-, subspecies- and species-specific epitopes that can be defined by monoclonal antibodies (Campbell et al. 1990, Kuo et al. 1995). The MOMP of *C. pneumoniae* is more homogenous and less immuno-
genic than that of the other chlamydiae (Campbell et al. 1990). Other outer membrane proteins, such as the cysteine-rich 60 kDa protein (Omp2) and the small cysteine-rich 12-15 kDa protein (Omp3) (Collet et al. 1989, Ting et al. 1995, Stephens et al. 2001), are present in smaller amounts. In addition, proteins called polymorphic outer membrane proteins (Pmps) have been localised in the outer membrane (Longbottom et al. 1998, Knudsen et al. 1999).

Chlamydial LPS, which is present in the outer membrane of both EBs and RBs, is an endotoxin generally found in gram-negative bacteria (Birkelund et al. 1989). Chlamydial LPS is structurally similar to the rough form of LPS found in enteric bacteria. It has both chlamydial genus-specific antigens and antigens shared by other members of the Enterobacteriaceae (Nurminen et al. 1983, Brade et al. 1987). However, the structure of chlamydial LPS is not identical in the different species, and the endotoxin activity of chlamydial LPS is much lower than that of enterobacterial LPS (Numminen et al. 1983, Brade et al. 1987, Ingalls et al. 1995). The proinflammatory cytokine response to C. trachomatis at the invasion phase is suggested to be mediated by LPS (Ingalls et al. 1995).

Several heat shock proteins (Hsp) have been found in chlamydial cell walls. The genes encoding Hsp10, Hsp60 and Hsp70 have been cloned and sequenced (Morrison et al. 1989a, Danilition et al. 1990, LaVerda & Byrne 1997). All three Hsps can be found in the outer membrane complex of both EBs and RBs (Brunham & Peeling 1994). These genes are continuously expressed throughout the developmental cycle. The Hsps are highly conserved within chlamydial species, including C. pneumoniae (Kikuta et al. 1991). Especially chlamydial Hsps 60, but also Hsp 70 and Hsp10 have been implicated as important agents in the immunopathology of chlamydial infections (Peeling & Mabey 1999a, LaVerda et al. 2000) (See 2.1.3., page 19).

Chlamydiae are obligate intracellular bacteria that occupy a non-acidified vacuole (inclusion) during their entire developmental cycle. These bacteria produce a set of proteins (Inc proteins) that localize to the surface of the inclusion within infected cells. The first of these proteins was demonstrated in C. psittaci by Rockey et al. (1995) and named IncA. Since then, six other Incs, from IncB to IncG, have been characterised (Bannantine et al. 1998, Scidmore-Carlson et al. 1999). However, a genome search of C. trachomatis revealed 46 candidates as potential members of Incs (Bannantine et al. 2000). The genes of C. pneumoniae contain an even higher number of hypothetical Inc proteins (Rockey et al. 2000).

Both C. trachomatis and C. pneumoniae genomes consist of a more than one million base pair (bp) chromosome that has been completely sequenced (Stephens et al. 1998, Kalman et al. 1999, also available at http://www.tigr.org). Most C. trachomatis strains have a~7500 bp plasmid. Systems for nutrient (amino acids, ions, etc.) transport from the host have been identified (Stephens et al. 1998, Kalman et al. 1999). Moreover, chlamydiae have a type III secretion system to interfere with host cell metabolism (Subtil et al. 2000). Comparison of the C. trachomatis and C. pneumoniae genomes will provide some knowledge of the common chlamydial biological processes required for infection and survival in mammalian cells and the difference between the two species in the disease spectrum.
2.1.3 Pathogenetic mechanisms of chlamydial infections

*Chlamydiae* are responsible for a wide variety of human and animal infections and have a tendency to cause recurrent and persistent or chronic infections (Schachter 1989, Kuo *et al.* 1995, Ward 1995). Different chlamydial species as well as different biovars infect different cell types. The trachoma biovars A-K of *C. trachomatis* infect primarily columnar but not squamous epithelium, causing mucosal infections, whereas the LGV strains of *C. trachomatis* as well as *C. pneumoniae* and *C. psittaci* are invasive (Schachter 1989). *In vitro* studies have shown the human biovar of *C. pneumoniae* to be able to infect and multiply in endothelial cells, smooth muscle cells, monocytes/magrophages and lymphocytes, which makes its systemic dissemination through circulation possible (Kaukoranta-Tolvanen *et al.* 1994, Gaydos *et al.* 1996, Fryer *et al.* 1997, Airenne *et al.* 1999, Haranaga *et al.* 2001).

The host defence mechanisms seem unable to eradicate *Chlamydia* or to provide complete protection from reinfection. Therefore, repeated infections with *Chlamydiae* are common (Grayston *et al.* 1985, Saikku 1992, Ward 1995). Repeated or persistent infections, which provide an opportunity for long-term stimulation of the host with chlamydial antigens, result in tissue damage (Beatty *et al.* 1994b, Ward 1995) and consequent adverse outcomes, in which Hsps seem to have a role (Beatty 1994a).

Heat shock proteins (Hsps) are highly conserved proteins present in all organisms ranging from bacteria to man. They are important antigens that induce both humoral and cell-mediated immunity. Hsps serve two major functions: firstly, under physiological conditions, they act as molecular chaperones upon the folding, unfolding, assembly and translocation of newly synthesized or damaged proteins (Lindquist 1986). Secondly, they are induced in response to cellular stress, which includes changes in temperature, the presence of free oxygen radicals, viral and bacterial infections, heavy metals, ethanol and ischemia (Lindquist 1986, Welch 1993, Zygel & Kaufman 1999a, b). Hsps are classified into four different families based on their molecular weight measured in kDa: Hsp90, Hsp70, Hsp60 and small Hsp10. Hsps are highly immunogenic: firstly, there is >50% sequence homology between prokaryotic Hsp and the Hsp of mammalian cells. Secondly, Hsps are immunodominant agents for many common microbes, which means that these infectious agents are mainly recognized by the immune system through recognition of their Hsp epitopes. Finally, Hsps are overexpressed at sites of acute and chronic inflammation (reviewed by Neuer *et al.* 2000).

Chlamydial Hsp60 (cHsp60) is expressed throughout the chlamydial developmental cycle (Engel *et al.* 1990, Shaw *et al.* 2000), and the amount of cHsp60 is increased after heat shock (Engel *et al.* 1990) and in other unfavourable growth conditions (Beatty *et al.* 1993 Beatty *et al.* 1994c). Chlamydial Hsp60 has been shown to elicit an ocular delayed hypersensitivity response (Morrison *et al.* 1989b), and its expression has been shown to be increased in persistent *C. trachomatis* infections (Beatty *et al.* 1994c). Because there is high amino acid sequence homology between microbial and human Hsps (Jones *et al.* 1993), the induced immune response against microbial Hsps may give rise to an autoimmune inflammatory reaction in the host.

An enhanced immune reaction against chlamydial Hsp60 is more typically associated with chronic upper genital tract conditions than with acute infections of the lower genital tract (Peeling & Mabey 1999a). Serum antibodies to chlamydial Hsp60 as well as Hsp-
specific T cell responses have been shown to be associated with blinding trachoma, salpingitis, pelvic inflammatory disease (PID), fallopian tube damage, ectopic pregnancy and TFI following ocular and genital C. trachomatis infections (Peeling & Mabey 1999a). Chlamydial Hsp10 is co-expressed with cHsp60 (LaVerda & Byrne 1997). The immune response is associated with the pathogenetic sequelae of chronic chlamydial infections (Betsou et al. 1999) and tubal factor occlusion (Spandorfer et al. 1999, LaVerda et al. 2000).

Chlamydial Hsp60 has been associated with the severity of the chronic sequelae of not only C. trachomatis infections but also other chlamydial infections. As regards diseases associated with C. pneumoniae infection, antibody responses to chlamydial Hsp60 in, for instance, arteriosclerosis (Ciervo et al. 2002, Mahdi et al. 2002, Huittinen et al. 2002) have been reported. Both human and chlamydial Hsp60 proteins have also been localized in atherosclerotic plaque macrophages (Kol et al. 1998). It has also been found that the Hsp60 of C. pneumoniae induces foam cell formation by inducing oxidation of LDL in monocytes (Kalayoglu et al. 1999).

2.1.4 Clinical spectrum of C. trachomatis infections

C. trachomatis infects primarily columnar and pseudostratified columnar epithelium, but not squamous epithelium, which makes it a pathogen of the mucosal surface (Black 1997).

2.1.4.1 Trachoma

Trachoma is the world’s leading cause of preventable blindness. The manifestations of ocular trachoma infections range from mild conjunctival lesions (follicular conjunctivitis) to severe forms that eventually lead to scarring and blindness. Severe forms develop through repeated or persistent infections by the C. trachomatis serovars A, B, Ba and C (Grayston & Wang 1975, Grayston et al. 1985, Abu el-Asrar et al. 2001). Therefore, trachoma is considered the prototype of chronic chlamydial infection. It has been estimated that about 500 million people have had the disease. In the developing countries, about 7 to 9 million people are estimated to be blind because of C. trachomatis infection (WHO, http://www.who.int/). Trachoma is endemic mainly in tropical and subtropical countries.

The main reservoir of the organism is the eye of an infected person, usually a child, and transmission may be potentiated by flies that carry infected secretion from person to person (Miller et al. 2004).
2.1.4.2 C. trachomatis infections in women

The genital chlamydial agent was first isolated from the cervix of a mother whose baby had neonatal inclusion conjunctivitis (Jones et al. 1950). However, knowledge of the diseases caused by genital tract infections with C. trachomatis only began to be gained in the 1970’s, after the development of cell culture techniques enabling isolation of the organism from urogenital samples (Grayston & Wang 1975).

Although C. trachomatis is still an important ocular pathogen in the developing countries, C. trachomatis studies have mostly focused on sexually transmitted infections, since the same organism that causes trachoma is considered the world’s most common sexually transmitted bacterial pathogen. According to the World Health Organization, 90 million new cases occur each year worldwide (WHO, http://www.who.int/). It has been estimated that, in many populations, about 10% of sexually active people are infected with C. trachomatis. The highest incidence of positive chlamydial culture is in the age group of less than 25 years. Since 1995, the incidence of C. trachomatis infection has been increasing in Finland. During 1995–2000, laboratory surveillance data documented an increase in the incidence rate from 23.4 per 10,000 to 29.2 per 10,000. The biggest increase in the incidence occurred in the youngest age group (10–19 years old). In 2000, 33.5% of the females with C. trachomatis were aged under 20 years (Hiltunen-Back et al. 2003). The incidence gradually decreases with increasing age (Grun et al. 1997, Fenton et al. 2001, Hiltunen-Back et al. 2001). Reinfections are also more common in women under 25 years of age than in older women (Xu et al. 2000, Burstein et al. 2001). Risk factors related to sexual behaviour include an increased number of sexual partners and a failure to use barrier contraceptives, such as condoms (Grun et al. 1997, Burstein et al. 2001, Fenton et al. 2001).

The clinical manifestations of C. trachomatis infection in women include acute urethral syndrome, urethritis, Bartholinitis, cervicitis, upper genital tract infection (endometritis, salpingo-oophoritis, or PID), perihepatitis (Fitz-Hugh-Curtis syndrome) and reactive arthritis. Genital C. trachomatis infection is asymptomatic in up to 80% of women (Stamm 1999), and only 20% of infected women present with symptoms.

The predominant C. trachomatis serotypes in urogenital infections are the serotypes D, E and F (Wang et al. 1985, Saikku & Wang 1987, van Duynhoven et al. 1998). Symptomatic genital tract infections in women have been suggested to be related to the chlamydial serotype G (Lan et al. 1995), whereas asymptomatic infections are often caused by the chlamydial serotypes D and F (Workowski et al 1994, Lan et al. 1995). Serotype E has been found in both symptomatic and asymptomatic women. Furthermore, Dean et al. (2000) showed that almost all patients with repeated C. trachomatis infections are infected with uncommon complex C serotypes, suggesting that the complex C is associated with chronic or recurrent infections. The relationship between different serotypes and syndromes is still debatable.

C. trachomatis is the major cause of mucopurulent cervicitis (Brunham et al. 1984). The patient may present with such symptoms as vaginal discharge, post-coital vaginal bleeding and mild abdominal pain. The initial site of infection is usually the cervix, but the urethra and rectum may also be involved (Stamm et al. 1980, Cates & Wasserheit 1991). Culture studies have shown that 50 to 60% of the women infected with C. trachomatis have cervical and urethral infections, 30% have only cervical infections and 5 to
30% have only urethral infections (Paavonen 1979, Paavonen et al. 1982, Phillips et al. 1987, Morris et al. 1993). Most women with chlamydial cervicitis or urethritis seem to have only mild symptoms or are completely asymptomatic (Paavonen 1979, Stamm et al. 1980, Cates & Wasserheit 1991). On examination, however, at least a third generally have local signs of infection such as endocervical bleeding, mucopurulent (green or yellow) endocervical discharge and edema within the area of ectopy (Paavonen et al. 1988).

*Chlamydia trachomatis* has also been associated with endometritis (inflammation of the endometrium) both in the presence and in the absence of salpingitis (Eckert et al. 2002).

Infection of the upper genital tract (e.g., endometritis, salpingitis) may be manifested as irregular vaginal bleeding and abdominal or pelvic discomfort (Greenwood & Moran 1981, Mårdh et al. 1981, Dieterle et al. 1998). A physical examination may reveal uterine tenderness or cervical motion tenderness, but a marked proportion of women have completely normal physical findings (Cadena et al. 1973).

*Chlamydia trachomatis* infection may persist subclinically in the endometrium for a long time (Paavonen et al. 1985a, b) and produce a chronic subclinical infection. It has been shown earlier using endometrial biopsies from women with suspected pelvic inflammatory disease that severe plasma cell endometritis and lymphoid follicles with transformed lymphocytes were significantly more common in a *C. trachomatis* culture-positive group than in a culture-negative group (Paavonen et al. 1985b). The presence of plasma cells in the endometrial stroma [i.e., plasma cell endometritis, PCE] seems to be characteristic of chronic endometritis (Greenwood & Moran 1981, Kiviat et al. 1990). The frequency of *C. trachomatis* as a causative agent in PCE has been reported to be between 18 to 52% (Kiviat et al. 1986, Paavonen et al. 1987, Paukku et al. 1999a). Severe PCE with lymphoid follicles has been shown to be significantly more common in endometritis caused by *C. trachomatis* than in non-chlamydial endometritis (Paavonen et al. 1985b). The natural history and long-term prognosis of endometritis is unknown, but it may occasionally progress to PID with consequent sequelae and subsequent infertility (Munday 2000).

PID has been defined as a syndrome associated with the spreading of microbes from the vagina and cervix to the endometrium, salpingeal tubes and adjacent structures (Weström 1980). Long-term complications of PID include ectopic pregnancy, TFI and chronic pelvic pain. The majority of PID cases are caused by *C. trachomatis*, *Neisseria gonorrhoeae* or both and anaerobic bacteria. Studies on the prevalence of *C. trachomatis* infection in patients with proven PID have shown that more than half of PID cases are caused by *C. trachomatis* (Paavonen & Lehtinen 1996). Seroepidemiological studies have indicated that chlamydial infections account for a large proportion of these asymptomatic cases by demonstrating a strong link between the presence of serum antibodies to *C. trachomatis* and the presence of tubal pathology (Punnonen 1979). Because *C. trachomatis* invades particularly columnar epithelium, the densely ciliated ampullary segment of the fallopian tube is most susceptible to chlamydial infection. In this way, chlamydial PID may cause distal tubal occlusion and subsequent infertility or partial distal occlusion with an increased risk for ectopic pregnancy. Women with PID have a 7- to 10-fold risk for ectopic pregnancy, and 43% of ectopic pregnancies may be due to chlamydial infections. Recent evidence suggests that ectopic pregnancy may be an acute or long-term complication of *C. trachomatis* infection (Egger et al. 1998). Chronic pelvic pain occurs in more than 15% of women with previous episodes of PID, and the incidence increases from 11%
after one episode to 66% after three or more episodes. It appears to correlate with the presence of peritoneal adhesions.

Several epidemiological studies have suggested that *C. trachomatis* infection is an independent risk factor for the development of cervical carcinoma (reviewed by Zenilman 2001). Additionally, specific serotypes of *C. trachomatis* have been linked to the risk of cervical cancer. In a large cohort of 530,000 Scandinavian women, Anttila *et al.* (2001) demonstrated that the *C. trachomatis* serotype G was most strongly associated with cervical cancer (OR=6.6, 95% CI 1.6–27). The serotypes I and D were also associated with cervical cancer, and the presence of multiple serotypes increased the risk. In another seroepidemiologic study from Finland, antibodies to the GFK serotype pool were more common in women who developed cervical squamous cell carcinoma than in controls (Lehtinen *et al.* 1996).

### 2.1.4.3 C. trachomatis infections during pregnancy

*C. trachomatis* is the sexually transmitted bacterial pathogen most likely to be found in an obstetric population, with 2–20% of pregnant women infected (McGregor & French 1991). In Finland, Gencay *et al.* (2000) found in their prospective study that mothers with preterm delivery (at 23–29 weeks of gestation) had a *C. trachomatis* IgM seropositivity rate of 8.3%. Untreated chlamydial infection during pregnancy is suggested to be associated with a number of adverse outcomes, including preterm labor, premature rupture of membranes, low birth weight, neonatal death and postpartum endometritis (Martin *et al.* 1982, Gravett *et al.* 1986, Martius *et al.* 1988, Andrews *et al.* 2000, Gencay *et al.* 2000). *C. trachomatis* may persist in the upper genital tract for months or even years (Shepard & Jones 1989), and the persistent infection in the endometrium may cause repeated adverse pregnancy outcomes. Early or recurrent pregnancy loss may be induced by asymptomatic *C. trachomatis* infection (Quinn *et al.* 1987, Witkin & Ledger 1992, Witkin 1999), although not all studies have revealed such a link (Paukku *et al.* 1999b). *C. psittaci* ovine abortion strain infection in humans is also suggested to cause abortion by inducing an acute inflammatory response in the placenta (Roberts *et al.* 1967, Johnson *et al.* 1985, Flanagan *et al.* 1996).

### 2.1.4.4 C. trachomatis infections in men

The clinical significance of *C. trachomatis* in non-gonococcal urethritis (NGU) and accessory sexual gland infection in men has been established during the past two decades (Paavonen & Eggert-Kruse 1999). NGU is the most common clinical genital syndrome seen in males, and *C. trachomatis* is the most important aetiological agent in it. Symptoms of NGU may develop after an incubation period of 7 to 21 days and include dysuria, mild-to-moderate whitish or clear urethral discharge or urethral pruritus. Up to 50% of infected men do not experience symptoms (Zellin *et al.* 1995). In most cases, physical examination reveals no abnormalities other than the discharge. Other clinical syndromes
in men include acute epididymitis, acute proctitis, acute proctocolitis, conjunctivitis, and Reiter’s syndrome. In young men, ‘idiopathic’ epididymitis is often caused by *C. trachomatis*. Unilateral scrotal pain is the primary symptom, and the common clinical signs of this infection include scrotal swelling, tenderness and fever. Chlamydial proctitis may occur in homosexual men (Black 1997, Stamm 1999). The prevalence of male chlamydial infection is dependent on age, number of sex partners and socioeconomic factors.

### 2.1.4.5 Lymphogranuloma venereum

LGV is a sexually transmitted systemic infection caused by the *C. trachomatis* serovars L1, L2 and L3. LGV is uncommon in industrialised countries but highly prevalent in parts of Africa, Asia and South America, and it occurs in both men and women. The LGV serovars of *C. trachomatis* are more invasive than the other genital serovars (Black 1997). It predominantly infects lymphatic tissue but may also occur as an acute symptomatic infection without apparent lymph node involvement or tissue reaction at the point of infection (Perine & Stamm 1999). Until 2003, LGV was considered a rare disease outside resource-poor countries. Since then, it has emerged as a significant problem among men who have sex with men in Europe. In 2003, an outbreak of LGV was recognised in the Netherlands (Niuewenhuis et al. 2004). Since that report, similar outbreaks have been seen in France. Cases have also been reported from Sweden and, more recently, from the United States and Canada. All the reported cases have been caused by the L2 serovar, although there is some evidence that a number of genetically distinct strains of *C. trachomatis* L2 are responsible for these outbreaks (Blank et al. 2005, French et al. 2005).

### 2.1.4.6 *C. trachomatis* infections in infants

*C. trachomatis* is the most common cause of neonatal conjunctivitis and one of the most common causes of pneumonia in early infancy. Infants of mothers with chlamydial infection will develop conjunctivitis in 18–50% and pneumonia in 11–20% of cases. Although these are thought to result from contact with infected vaginal secretions, there have been reported cases where neonatal chlamydial infection was found in infants delivered by caesarean section in the presence of intact membranes (Ratelle et al. 1997). Symptoms of conjunctivitis usually develop within 2 weeks after delivery, and if the infection is untreated, chlamydial pneumonia develops in 4 to 17 weeks after delivery. A very early onset suggests that the infection may even start as an intrauterine chlamydial infection (Mårdh et al. 1984, Gencay et al. 2000).

Neonatal chlamydial pneumonia has correlated with low birth weight. Infants with chlamydial pneumonia are at an increased risk to develop chronic obstructive lung disease later in childhood. Serological findings suggest a correlation between chronic lung disease and intrauterine chlamydial infection in extremely low-weight prematurely born infants. (Reviewed by Mårdh 2002.)
2.1.5 Clinical spectrum of *C. pneumoniae* infections

*C. pneumoniae* infections occur worldwide both endemically and epidemically, and the prevalence varies from one region to another. *C. pneumoniae* is primarily transmitted from human to human by the respiratory tract without any animal reservoir (Saikku *et al.* 1985, Kleemola *et al.* 1988). The incubation time is several weeks, which is longer than that for many other respiratory pathogens (Kuo *et al.* 1995).

*C. pneumoniae* infections occur annually, but cyclic variations have been shown in the incidence: 2- to 3-year periods of high incidence are followed by 4- to 5-year periods of low incidence (Schachter & Grayston 1998). *C. pneumoniae* infections appear to be most common among school-aged children in developed countries (Kuo *et al.* 1995). The prevalence increases dramatically after the age of 5, and by the age of 20, half of the population are estimated to have detectable antibody levels. Unlike *C. trachomatis*, *C. pneumoniae* antibody prevalence is higher in males than in females (Saikku 1992). Seropositivity with *C. pneumoniae* antibodies continues to rise steadily in the population along with age and reaches a level of approximately 75% in the elderly, while in the case of *C. trachomatis*, antibody prevalence clearly decreases after 40 to 50 years of age. This indicates that the majority of people get infected by *C. pneumoniae* during their lifetime, and that reinfections are common (Grayston *et al.* 1990, Saikku 1992, Kuo *et al.* 1995). In addition, smoking has been shown to be associated with *C. pneumoniae* seropositivity in the general population (Karvonen *et al.* 1994).

*C. pneumoniae* is a respiratory pathogen that causes both upper and lower respiratory tract diseases. The majority of *C. pneumoniae* infections are asymptomatic or mild upper respiratory tract infections (Saikku 1992, Miyashita *et al.* 2001). Several chronic respiratory tract inflammatory diseases have also been associated with *C. pneumoniae* infection. These include chronic bronchitis, chronic obstructive pulmonary disease (COPD), sarcoidosis, onset of asthma, asthma exacerbations (Kuo *et al.* 1995, von Hertzen *et al.* 1996, Hahn & Allegra 1999, Saikku 2002) and even lung cancer (Laurila *et al.* 1997, Jackson *et al.* 2000, Koyi *et al.* 2001).

In addition to respiratory tract infections, *C. pneumoniae* has been associated with cardiovascular diseases. Subacute inflammatory conditions, such as endocarditis, myocarditis and vasculitis, have been reported to follow *C. pneumoniae* infections (reviewed in Saikku 2002). The association of *C. pneumoniae* infection with coronary heart diseases and acute myocardial infarction was discovered in 1988 by Saikku *et al.* (1988). Although no causal association between *C. pneumoniae* infection and atherosclerosis has been demonstrated, up to 500 papers have been published to support the theory that *C. pneumoniae* infection is involved in the clinical diseases associated with atherosclerosis and its complications, such as acute myocardial infarction, stroke, transient ischaemic attack and abdominal aneurysm (reviewed in Ngeh *et al.* 2002). The evidence is still controversial to some extent.
2.1.6 Diagnosis of chlamydial infection

The methods currently used to diagnose chlamydial infections include serological tests, isolation by cell culture, direct detection of chlamydial antigens and amplification of chlamydial DNA.

2.1.6.1 Serology

The following serologic tests have been developed for the diagnostic detection of chlamydial antibodies: complement fixation (CF) test, microimmunofluorescence (MIF) test and EIA.

The first widely used serological test was the complement fixation test. The CF test detects antibodies against chlamydial genus-specific LPS. It is therefore unable to differentiate between the species. The CF test is a relatively insensitive method to detect antibodies elicited in C. trachomatis infections, and it can only be used to diagnose LGV, the systemic infection caused by the C. trachomatis serotypes L1-L3. It is of little value in the diagnosis of oculo-genital chlamydial infections. Most patients with proven C. trachomatis infection of the male urethra or the female cervix do not express CF antibody levels >1:16 (Black 1997). In infant chlamydial infections, the CF test is not useful because LPS is a poor immunogen in infants (Puolakkainen et al. 1984). Both IgG and IgM class antibodies react in the CF test, but this test does not differentiate between IgG and IgM antibodies. Although lacking in specificity, the CF test is technically much less demanding than MIF and has objective endpoints. Another thing in favour of the CF test is that LPS antibodies are produced very early in primary infection. The sensitivity of the CF test in primary respiratory infection is about 60%. In reinfections, however, the CF test is not suitable: complement-fixing LPS antibodies are rarely detectable by the CF test, the sensitivity being only 10%. (Black 1997, Peeling 1999b).

The MIF test developed by Wang & Grayston was initially used for serotyping chlamydial strains (Wang & Grayston 1970). The MIF test measures antibodies against chlamydial EB antigen, and the test is able to differentiate both species- and serotype-specific antibodies. It is able to measure separately antibodies in the IgA, IgM and IgG classes, and it is therefore suitable for distinguishing recent from past infections as well as primary from reinfections (Wang & Grayston 1970, Kuo et al. 1995). If performed and read properly, this test provides a sensitive and the most specific method for the laboratory diagnosis of chlamydial infections. In the case of acute chlamydial infection, the criterion for a serological diagnosis is a fourfold rise in the IgG or IgA titre or a single IgM titre of ≥ 16 for both C. pneumoniae and C. trachomatis (Kuo et al. 1995, Black 1997). The criteria for seropositivity using MIF are shown in Table 2. However, as an acute C. pneumoniae infection usually induces high levels of IgG antibodies by MIF, the same phenomenon is infrequently seen in infections with other chlamydial species (Grayston et al. 1990, Kuo et al. 1995, Black 1997). In addition, the need for paired sera to show a fourfold rise in IgG titre and the fact that the IgG antibody response may occur 6–8 weeks after the onset of illness, limit the use of MIF in the diagnosis of primary infections.
Both elevated short-lived IgA antibodies and microbe-specific circulating immune complex (IC) have been shown to persist in chronic *C. pneumoniae* infections (Saikku 1992, Saikku 1999; Table 2). ICs consist of microbial antigens and antibodies produced in defence against pathogens. Their presence in the circulation is a sign of continuous production of microbial antigens in the close vicinity of the vascular system, and their presence is hence a potential marker for persistent infection. The presence of circulating ICs is typical for many chronic viral and bacterial diseases (reviewed by Saikku 1999).

**Table 2. Criteria for serodiagnosis of chlamydial infections by MIF.**

<table>
<thead>
<tr>
<th>Chlamydial infection</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute infection</td>
<td>IgM titre 16</td>
</tr>
<tr>
<td></td>
<td>Fourfold rise in IgG titre</td>
</tr>
<tr>
<td></td>
<td>Fourfold rise in IgA titre</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>Persistent presence of elevated IgG and/or IgA antibodies and/or ICs</td>
</tr>
</tbody>
</table>

EIA is also able to differentiate between the three antibody classes. EIA kits with EBs of *C. pneumoniae* or *C. trachomatis* as antigen are commercially available for the detection of chlamydial antibodies. EIA tests are generally preferred in serology due to their simplicity and possibility for automation (reviewed by Peeling 1999b). Recently, EIA tests that apply *C. trachomatis* MOMP variable domain IV synthetic peptides as antigen have been developed (Närvänen *et al.* 1997). Because EIA tests based on synthetic peptides are also antigen site specific (Norrby *et al.* 1987), they can discriminate antibody responses even against different chlamydial immunotypes (Jones *et al.* 1992).

Although MIF is still considered the gold standard, new EIA tests hold the promise to become useful alternatives to MIF. Comparisons between MIF and EIA tests have been published. One recent comparison of this kind showed sensitivities between 71 and 85% and specificities of more than 96% for three different EIA kits (Morré *et al.* 2002). In another study, antibodies to *C. trachomatis* were detected by EIA in 84% of culture-positive women and in 61% of culture-positive men. Titre changes were observed in a minority of the cases (Närvänen *et al.* 1997). There are only a few studies available in which *C. trachomatis* antibodies measured by EIA and MIF have been compared in predicting tubal pathology, and no significant differences between peptide-based EIAs and the MIF have been found (Gijsen *et al.* 2002, Bax *et al.* 2003).

EIA tests have also been developed for the measurement of antibodies against *C. pneumoniae*. However, problems with sensitivity and specificity have been encountered. As in the case of *C. trachomatis*, if the absorbance threshold is raised to increase the specificity of the assay, sensitivity decreases, and vice versa (reviewed by Peeling 1999b).
2.1.6.2 Culture

Until recently, culture was considered the golden standard for the detection of *C. trachomatis* in urogenital specimens because it has specificity that approaches 100% but is relatively insensitive: its sensitivity is 50%–85% compared to nucleic acid amplification tests (reviewed by Black 1997). Other disadvantages when using culture are the requirement for careful transportation, the requisite high-level technical expertise and time-consuming incubation (3–7 days). Since culture detects only viable infectious chlamydial elementary bodies and has minimal potential for contamination, it has still remained the standard for medico-legal purposes (reviewed by Black 1997, Caul & Herring 2001). Although *C. trachomatis* is relatively easy to culture in acute infections, the isolation of *C. pneumoniae* by culture is more difficult. The sensitivity of cell culture in the diagnosis of acute *C. pneumoniae* respiratory infection is approximately 60% compared to serology, while specificity is 100%. However, isolation from the chronic stage is much more difficult (reviewed by Kuo 1999, Saikku 1999).

2.1.6.3 Antigen detection

The antigen detection methods based on the demonstration of genus-specific chlamydial LPS cannot differentiate between chlamydial species (Black 1997, Peeling 1999b). Commercial LPS-EIA methods designed for the detection of *C. trachomatis* can also be used for the detection of the *C. pneumoniae* antigen, since the target of the capture antibody in chlamydial EIA kits is a genus-specific LPS. The performance of these assays has not been extensively evaluated (Black 1997). Antigen detection by EIA, however, is considered more sensitive than culture in chronic *C. trachomatis* infections (Saikku 1994, Black 1997).

In the direct fluorescence antibody technique (DFA), polyclonal or monoclonal antibodies with a fluorescent label are used to detect chlamydia in infected cells in smears made from a variety of specimens. The method was widely used in the diagnosis of *C. trachomatis* infections before the more sensitive nucleic acid amplification methods became available (Lipkin *et al.* 1986). Although a commercial DFA method has also been developed for the diagnosis of respiratory infections caused by *C. pneumoniae* infections, it has been used only occasionally due to its rather poor sensitivity and difficult interpretation (P. Saikku, personal communication).

2.1.6.4 Nucleic acid amplification

The development of tests based on the nucleic acid amplification technology has been the most important advance in the field of chlamydial diagnosis. Since all nucleic acid amplification technologies detect nucleic acid targets, they do not depend on the viability of the target organism. The fact that nucleic acid amplification is exquisitely sensitive and high-
ly specific offers the opportunity to use also non-invasive sampling techniques. Nucleic acid amplification tests have been used to detect *C. trachomatis* in first-void urine specimens and vaginal swabs (Schachter *et al.* 1995, Stary *et al.* 1997, Black & Morse 2000) and *C. pneumoniae* in sputum (von Hertzen *et al.* 1997), circulating, purified white blood cells (Boman *et al.* 1998) and tissues (Kuo *et al.* 1995).

The most widely known of DNA amplification technology is PCR. The specificity of the PCR method compared to culture is 95–100% for both *C. pneumoniae* and *C. trachomatis* (Black 1997, Peeling 1999b). Due the inhibitory factors present in samples, the sensitivity of PCR has been variable. However, it has been estimated that PCR, in general, is at least 25% more sensitive than culture (Kuo *et al.* 1995, Black 1997, Peeling 1999b). Ligase chain reaction (LCR) is another commercially available nucleic acid amplification technology used for the diagnosis of *C. trachomatis* (Dille *et al.* 1993). Including the most commonly used PCR, LCR, such as transcription mediated amplification (TMA) assay, and strand displacement amplification (SDA) assay based diagnostic tests are commercially available for diagnostic and research purposes (Caul & Herring 2001). Recently, a quantitative real-time PCR technique has also been developed for the detection of *C. pneumoniae* (Mygind *et al.* 2001).

### 2.1.7 Treatment

**Acute infections.** Many randomized controlled trial have demonstrate that doxycycline, erythromycin and azithromycin are equally effective in the treatment of *C. trachomatis* infections in non-pregnant women. Clinical success also depends on the patients’ adherence with the effective antibiotic regimen, sexual abstinence during therapy and treatment sexual partners (Clarke 2001). In the choice of therapy, the main advance of single-dose azithromycin is that it guarantees virtually 100% compliance. Cephalosporin-based combinations for treating suspected pelvic infections should be avoided, because they do not effectively cure chlamydial infections (Clarke 2001). In a detailed study of women treated with -lactam antibiotics for acute salpingitis, Sweet *et al.* (1983) found persistence of endometrial and cervical infection despite the completion of antibiotic regimens and clinical improvement. Inappropriate antibiotic treatment may also lead to chronicity of the disease. Brunham *et al.* (2005) found an unexpected impact of a *C. trachomatis* infection control program on susceptibility to reinfection. There is wide experience erythromycin, amoxicillin and clindamycin medication during pregnancy. Data on the safety of azithromycin use during pregnancy are limited. However, azithromycin has been widely used in routine obstetric practice during the past decade, and no serious adverse outcomes related with its use have so far been reported (Genc 2002).

A number of different antibiotics have been tested in search for an appropriate treatment for *C. pneumoniae* infection. Azithromycin and clarithromycin are two macrolides, which have high activity against the organism *in vitro* (Agacfidan *et al.* 1993, Weber & Johnson 1995). Some of the new fluoroquinolones have also turned out effective (Miyashita *et al.* 2002). *C. pneumoniae* is not susceptible *in vitro* to sulpha drugs, and penicillin and ampicillin prevent the growth of the organism, but do not destroy it (Kuo *et al.* 1995). Clinical experience has shown that the symptoms of *C. pneumoniae* infection frequently
recur after short or conventional courses of appropriate antibiotics, and intensive long-term therapy is therefore highly recommended (Kuo et al. 1995).

**Chronic infections.** Treatment of *C. trachomatis* infections may be problematic since treatment failures are often reported, possibly due to the ability of chlamydiae to trigger persistent infections not responsive to antimicrobial treatment. Antibiotics used for treatment may in themselves also cause persistence of the infection (Johnson & Hobson 1977, Ridgway et al. 1978). *In vitro*, persistent *C. trachomatis* infections can be established by treatment with gamma interferon (IFN-γ) (Beatty et al. 1993) or penicillin (Clark et al. 1982) or by deprivation of certain nutrients (Harper et al. 2000). Treatment of chronic *C. trachomatis* with azithromycin should be a particularly efficacious anti-infective regime for the eradication of IFN-gamma-induced chlamydial persistent infection *in vivo* (Reveau et al. 2005). Treatment of chronic *C. trachomatis* and *C. pneumoniae* infections with multiple antibiotics has been suggested as well, since synergistic effects of antibiotics have been reported *in vitro* (Freidank et al. 1999). However, chronic or persistent *C. pneumoniae* infection does not respond to antimicrobial treatment, implicating that neither chronic infections nor advanced atherosclerotic disease can be treated with antimicrobial monotherapy. (Higgins 2003, Andraws et al. 2005, Danesh 2005)

### 2.2 CRP – a marker of inflammation and infection

C-reactive protein (CRP) is a pentameric hepatocyte protein with a half-life of 15 to 19 hours (Vigushin et al. 1993), and it is the major marker of the “acute-phase response,” or the formation of plasma proteins in response to an inflammatory stimulus, in humans (Morley & Kushner 1982). Synthesis of CRP in the liver is largely controlled by interleukin (IL)-6 and also by tumour necrosis factor-alpha (TNF-α) and IL-1 (Castell et al. 1990).

CRP shows no diurnal or seasonal variation, nor does it correlate with nutritional status or sex in healthy populations (Vigushin et al. 1993, Meier-Ewert et al. 2001). Smoking and obesity correlate positively with CRP levels, and weight loss and cessation of smoking reduce CRP values (Danesh et al. 1999, Yudkin et al. 1999, Rohde et al. 1999).

Both acute total and partial sleep deprivation resulted in elevated hsCRP concentrations (Meier-Ewert et al. 2004). Frequent physical activity is associated with low CRP levels. This may indicate a lower level of systemic inflammation in those engaged in physical activity compared with those not engaged in any physical activity (Ford 2002).

In the mid-1990s, immunoassays with greater sensitivity revealed that CRP values less than 10 mg/l, which were previously considered normal in everyday clinical practice, can be associated with infection activity. The recent advent of high-sensitivity technology permits the measurement of CRP levels as low as 0.007 mg/l compared to the previous detection limits of 3 to 5 mg/l (Rifai et al. 1999). The median CRP value in the general population (5748 adults from two study cohorts, age 25–74 years) rose with age (Hutchinson et al. 2000). For example, the mean CRP concentration approximately doubled with age, from 1.13 mg/l among men aged 35–44 years to 1.93 mg/l in men aged 65–74 years (Hutchinson et al. 2000). The corresponding CRP values in women were 1.08 mg/l and 2.22 mg/l. Epidemiological studies point to CRP as a predictor of both a long- and short-
term risk of stroke and myocardial infarction in men and women (Danesh et al. 2000, Ridker 2001). CRP has been shown to promote the secretion of inflammatory mediators by vascular endothelium (Lagrant et al. 1999) and to opsonize low-density lipoprotein for uptake by macrophages in atherosclerotic plaque (Zwaka et al. 2001). These data suggest that CRP may be directly implicated in the development of atherosclerotic lesions.

Elevated CRP concentrations are associated with the development of symptomatic peripheral arterial disease (Ridker et al. 1998). Elevated CRP is also an independent predictor of insulin resistance syndrome (Festa et al. 2000) and type 2 diabetes in apparently healthy women (Hu et al. 2004). However, at present, we do not have enough information of the role CRP in the pathogenesis of vascular diseases in females (Lawlor et al. 2005).

2.3 Chronic infections and infertility

2.3.1 Causes of infertility

A couple is said to be infertile if pregnancy is not achieved within one year despite regular intercourse (Barbieri 1999). Infertility is present in 10–15% of all couples (Barbieri 1999). Typically, 80% of couples (aged 18–28 years) will conceive over a one-year period and another 10% will conceive during the following year. At present, almost 80% of infertile couples can be helped to have a child. The incidence of voluntary infertility is very low, approximately below 3% (Templeton et al. 1991). Term subfertility is also used to describe impaired fertility. The time to pregnancy in months (TTP, the time elapsed from the date the couple have started trying to conceive to the time the woman becomes pregnant) approach is the best-known measure of infertility at the population level, and it is widely used as a definition of subfertility (Joffe et al. 1993, 1995). The rate of infertility has been slowly increasing (Stephen & Chandra 1998, Silverberg 2000). The reasons for the rising incidence of infertility include postponement of childbearing, increased pelvic infections and, in some countries, deteriorating sperm quality (Unkila-Kallio 2001).

One third of instances of infertility are related to a female factor, one third to a male factor, and one third to a combination of the two. The most frequent cause of infertility is anovulation, followed by a male factor and tubal occlusion/abnormalities (Table 3). However, in spite of thorough examinations, the cause of infertility still remains unexplained in approximately 10–20% of infertile couples (Collins 1995).


<table>
<thead>
<tr>
<th>Cause of sub/infertility</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anovulation</td>
<td>27</td>
</tr>
<tr>
<td>Tubal factor</td>
<td>22</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>5</td>
</tr>
<tr>
<td>Uterine/cervical factor</td>
<td>4</td>
</tr>
<tr>
<td>Male factor</td>
<td>25</td>
</tr>
<tr>
<td>Unexplained</td>
<td>17</td>
</tr>
</tbody>
</table>
2.3.2 Infections in females

Pelvic inflammatory disease (PID) is the major cause of TFI. PID is usually the result of infection ascending from the endocervix and causing endometritis, salpingitis, parametritis, oophoritis, tuboovarian abscess and/or pelvic peritonitis. The most important causative agents are *C. trachomatis*, *N. gonorrhoeae* and microbes associated with bacterial vaginosis. Studies on the prevalence of *C. trachomatis* infection in patients with proven PID have shown that more than half of PID cases are caused by *C. trachomatis* (Paavonen & Lehtinen 1996). Weström’s classic studies (1980) indicate that the incidence of TFI is approximately 10% after one episode of PID, about 20% after two episodes and over 40% after three episodes. Most women with TFI have never been diagnosed with PID. A large proportion of *C. trachomatis* infections of the Fallopian tubes are asymptomatic or subclinical. This suggests that silent infections are actually the most common causes of TFI (Paavonen & Eggert-Kruse 1999).

In addition, it has been hypothesized that chronic inflammatory bowel disease might at least (ulcerative colitis and Crohn’s disease) reduce fertility (Hudson et al. 1997, Sela et al. 2005). Ulcerative colitis is a chronic inflammatory bowel disease, and the onset peaks at the age of 15–30 years and coincides with the reproductive period. The presence of ulcerative colitis per se was not associated with decreased reproductive capacity. However, associated conditions, such as active disease, surgical interventions and medication, may interfere with reproduction. (Sela et al. 2005)

2.3.3 Infections in males

An inflammatory disease has been established also to affect male reproductive function and fertility, but little is known about the role of chronic Chlamydia infections in male infertility. Relevant inflammatory diseases include general and chronic infectious diseases as well as localized acute or chronic infections of the male genitourinary tract. A history of urogenital infection occurs in 5–12% of men attending infertility clinics (Gonzalez et al. 2004).

Usually, infection has a detrimental effect on sperm quality by reducing concentration and motility and possibly affecting the number of morphological normal spermatozoa. There is no convincing evidence for a relationship between previous chlamydial infection and semen quality (Paavonen & Eggert-Kruse 1999). However, there is in vitro evidence that the function of human spermatozoa can be significantly affected by direct exposure to *C. trachomatis*, which leads to premature sperm death evidently due to chlamydial LPS (Hosseinzadeh et al. 2003). Co-incubation of sperm with *C. trachomatis* LPS also results in cellular death partly due to apoptosis (Eley et al. 2005a). These findings provide an explanation of how *C. trachomatis* could mediate premature death of human sperm. In addition, chlamydial infections in males are of concern because of the risk of microbial transmission microbes to female partners, resulting in PID, ectopic pregnancy or infertility (Paavonen & Eggert-Kruse 1999). Infection may also be the source of auto-antibodies against spermatozoa, which are found in about 8% of the infertile male population (Gonzalez et al. 2004).
A significant impairment of sperm production occurs in patients with orchitis, whether due to mumps virus infection or a bacterial infection. Total sterility may result from a severe attack of bilateral mumps orchitis. In other situations, sperm production recovers after successful treatment of epididymoorchitis. In some individuals, recurrent attacks of epididymoorchitis result in progressive depletion of the germ cell complement and consequent severe oligospermia.

In contrast to the situation in women, there is no clear evidence showing that male accessory gland infections can result in epididymal blockade or vassal obstruction, with the exception of genital tuberculosis (Gonzalez et al. 2004). Although C. trachomatis is a well-documented source of chronic prostatitis, the infection does not seem to cause obstruction of the reproductive tract, as it does in women (Black 1997, Stamm 1999). If a male urogenital infection causes obstruction, it is most likely to occur at the level of the ejaculatory ducts.

The relationship between chronic prostatitis and male accessory gland infection and infertility has been controversial for many years. Bacteria, viruses, leukocytes, reactive oxygen species, cytokines, obstruction and immunological abnormalities have been suggested as co-factors in the development of infertility in patients with male accessory gland infection and prostatitis (Everaert et al. 2003). Chronic prostatitis has been shown to cause scarring of the prostatic and ejaculatory ducts, resulting in low seminal volume. Many of these men present with severe oligozoospermia or azoospermia, but normal testicular size and normal gonadotropins (Hales et al. 1999, Diemer et al. 2003, Dohle 2003, Gonzalez et al. 2004).

Infection, trauma, allergy, neurogenic damage and chemical or mechanical factors may lead to a long-lasting inflammation of the prostate or pelvic organs, even after eradication of the aetiologial agent, and be potentially related to infertility through cytokines (Everaert et al. 2003).

It has long been recognized that chronic inflammation and systemic infection are associated with decreased reproductive capacity. Men with critical illness, burn trauma, sepsis and rheumatoid arthritis are reported to have markedly reduced serum testosterone levels, resulting in at least temporary infertility (reviewed by Hales et al. 1999).

### 2.4 Role of inflammation in preterm delivery

Prematurity is one of the most significant problems confronting obstetrician in industrial countries today. Preterm births, defined as those occurring at less than 37 week’s gestational age, cause approximately 75% of perinatal mortality and as much as 50% of long-term neurologic handicap (Amon 1999).

An estimated 50% of spontaneous preterm births are associated with ascending genital tract infection (Lockwood 2002), and those occurring before 30 weeks’ gestation are even more likely to be infection-related (Russel 1979, Romero et al. 1989a, Watts et al. 1992). Infections at multiple sites are associated with spontaneous preterm birth: (1) intrauterine infection, either overt or subclinical; (2) lower genital tract infection colonization; and (3) distant infections, such as periodontitis.
Intrauterine infections are often chronic and usually asymptomatic until labour or the rupture of membranes. Even during labour, most women who are later shown to have chorioamnionitis (by histologic finding or culture) have no other symptoms except preterm labour. Histologically confirmed bacterial infections within the uterus, which may occur between the maternal tissue and the fetal membranes (chorioamnionitis), within the placenta (villitis), within the amniotic fluid (amnionitis) or within the umbilical cord of the fetus (funisitis), have consistently been linked with prematurity, low birth weight and premature rupture of membranes (Goldenberg et al. 2000). Bacterial infections have been detected in 19% to 74% of placentas in preterm deliveries and in 4% to 16% in term deliveries (Russell 1979, Mueller-Heubach et al. 1990). More information was obtained from the study by Hillier et al. (1988), which demonstrated that either histologic chorioamnionitis or the recovery of bacteria by culture of the placenta and foetal membranes was associated with preterm birth, but that a combination of the two was associated most strongly.

Studies evaluating amniotic fluid cultures in the setting of preterm labour (PTL) with intact membranes have revealed a prevalence of intra-amniotic infection ranging from 0% to 2% (Gibbs et al. 1992). Many infections are polymicrobial, and the most common organisms isolated are genital mycoplasma (Ureaplasma urealyticum and Mycoplasma hominis), anaerobes, group B streptococci, Gardnerella vaginalis and gram-negative rods, including Escherichia coli (Romero et al. 1989a, 1993, Hillier et al. 1988). It has recently also become evident that intrauterine infection with C. trachomatis can occur even in mothers with intact membranes (Gencay et al. 2000).

By using more sensitive assays, such as PCR, bacteria can be detected in the amniotic fluid of 30% to 50% of patients in preterm labour (Hitti et al. 1997, Oyarzun et al. 1998, Markenson et al. 1997). Although PCR is very sensitive and rapid, the clinical significance of the presence of bacteria in the amniotic fluid shown by PCR remains unclear.

More recently, using IL-6 as a marker of infection, it has been observed in several series that women undergoing routine genetic amniocentesis at 16 to 18 weeks and found to have high amniotic fluid IL-6 levels will frequently deliver at less than 32 weeks (Ghidini et al. 1997, Wenstrom et al. 1996, 1998) Using vaginal or cervical foetal fibronectin as a marker for intrauterine infection, it has been observed that women who are positive for this marker at 24 weeks of gestation already will often have a spontaneous preterm delivery (PTD) with clinical or histologic chorioamnionitis an average of 7 weeks later (Goldenberg et al. 1996).

Lower genital tract infections and preterm delivery

The hypothesis that ascending lower genital tract infection leads to preterm labour has been supported by multiple in vivo and in vitro studies (Keelan et al. 2003, Romero et al 2002, Goldenberg et al. 2000). The entry of lower genital tract bacteria into the decidua is associated with recruitment of leukocytes followed by cytokine production (Keelan et al. 2003). Cytokines have been found to trigger prostaglandin synthesis in the amnion,
chorion, decidua and myometrium (Keelan et al. 2003). This, in turn, leads to uterine contractions, cervical dilatation, membrane exposure and enhanced entry of microbes into the uterine cavity. Lower genital tract bacteria may also act locally, producing enzymes, such as sialidase or mucinase, which may weaken the protective cervical mucus and promote bacterial invasion into the upper genital tract (McGregor et al. 1994).

Bacterial vaginosis (BV) is associated with a two-fold risk of PTD, the greatest risk occurring when BV is present before 16 weeks of gestation (OR 7.55) (Leitich et al. 2003). This may indicate a critical period during early gestation when BV-related organisms can gain access to the upper genital tract and set the stage for PTL later in gestation (Kekki 2002). There is also evidence of an association between BV and chronic intrauterine plasma-cell endometritis in nonpregnant women: it is thus possible that intrauterine colonization associated with spontaneous PTL is present at conception (Korn et al. 1995).

Syphilis (Fiumara 1952) and untreated gonorrhoea (Elliot et al. 1990) are associated with PTD, and Trichomonas vaginalis infection has been associated with the significant increase of low birth weight (Kigozi et al. 2003). PTD has also been associated with abnormal vaginal flora other than BV, including aerobic bacteria, e.g. E. coli, group B streptococci and Staphylococcus aureus (reviewed by Goldenberg et al. 2000). Vaginal colonization with Candida species and Ureaplasma urealyticum has been studied and found to be unrelated to the increased risk of PTD (Carey et al. 1991, Cotch et al. 1998).

C. trachomatis infections have been associated with preterm delivery as well as with premature rupture of the membranes (PROM). Overall, however, there are only a few well-designed and controlled studies on the role of C. trachomatis in PTD/PROM, and these studies are summarized in Table 4. Unfortunately, randomized trials of treatment of C. trachomatis during pregnancy have not demonstrated a consistent reduction in the rate of preterm birth (Martin et al. 1997). Screening and treatment for C. trachomatis in all pregnant women is recommended, however, to reduce vertical transmission and spreading of sexually transmitted disease (Centers for Disease Control and prevention 2002). The role of chronic C. trachomatis infection in PTD is not known.
Table 4. Summary of studies on Chlamydia trachomatis (Ctr) infections and premature rupture of the membranes and preterm delivery.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study design</th>
<th>Sample size</th>
<th>Controls</th>
<th>Method</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Martin <em>et al.</em> 1982</td>
<td>prospective study</td>
<td>n=268 gravid women</td>
<td>n=234 culture-negative, matched for age, marital and socioeconomic status, pregnancy order, race.</td>
<td>endocervix culture &lt; 19 gw</td>
<td>PTD (5/18) 28% culture-positive versus (15/234) 6% culture-negative</td>
</tr>
<tr>
<td>Andrews <em>et al.</em> 2000</td>
<td>prospective study</td>
<td>n = 190 spontaneous preterm birth at &lt;37 gw</td>
<td>n = 190 delivery 37 gw matched for race, parity, and center</td>
<td>urine LCR at 24 and 28 gw</td>
<td>PTD at &lt;37 gw OR 2.2 (95% CI 1.03-4.78) and at &lt;35 gw OR 3.2 (CI 95% 1.08-9.57) infected versus uninfected</td>
</tr>
<tr>
<td>Gencay <em>et al.</em> 2000</td>
<td>prospective study</td>
<td>n=48</td>
<td>serum MIF</td>
<td></td>
<td>Ctr IgM positivity-rate high among PTD mothers (8.3%).</td>
</tr>
<tr>
<td><strong>PROM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harrison <em>et al.</em> 1983</td>
<td>prospective study</td>
<td>n=1365 Ctr antibodies 8.0%</td>
<td>endocervix culture Serum MIF at first prenatal visit and third trimester.</td>
<td></td>
<td>PROM (7/17) IgM-seropositive than either (453; \chi^2 = 8.60, p=0.01) IgM-negative or (74/790 \chi^2 = 15.29, p&lt;0.001) culture-negative women.</td>
</tr>
<tr>
<td>Grawett <em>et al.</em> 1986</td>
<td>prospective study</td>
<td>n=534 gravid women</td>
<td>endocervix culture second and third trimester.</td>
<td></td>
<td>PROM OR 1.5 (95% CI, 0.8-2.0) culture-positive versus culture-negative.</td>
</tr>
<tr>
<td>Sweet <em>et al.</em> 1987</td>
<td>prospective case-control study</td>
<td>n=270 matched for age, race, and socioeconomic status</td>
<td>endocervix culture serum MIF prenatal and at 30–34 gw</td>
<td></td>
<td>PROM and PTD RR 2.4 (p=0.02) IgM-positive versus IgM-negative</td>
</tr>
<tr>
<td>Alger <em>et al.</em> 1988</td>
<td>prospective case-control study</td>
<td>n=53 PROM &lt;37 and &gt; 20 gw</td>
<td>n=84 matched for age, parity, and gestational age</td>
<td>endocervix culture</td>
<td>PROM OR 4.33 (p=0.001), culture-positive versus culture-negative.</td>
</tr>
<tr>
<td>Reference</td>
<td>Study design</td>
<td>Sample size</td>
<td>Controls</td>
<td>Method</td>
<td>Outcome</td>
</tr>
<tr>
<td>----------------------</td>
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</tr>
<tr>
<td>Ryan et al. 1990</td>
<td>prospective study</td>
<td>n=11 544</td>
<td>culture-positive n=2433, culture-positive and treated n=1323</td>
<td>endocervix culture at first prenatal visit.</td>
<td>PROM OR 2.12 (95%CI 1.57-2.86) untreated positive versus negative</td>
</tr>
<tr>
<td>Ekwo et al. 1993</td>
<td>case-control study</td>
<td>n=463</td>
<td>preterm PROM, full-term PROM preterm without PROM</td>
<td>endocervix culture</td>
<td>Preterm PROM OR 5.0 (95%CI 1.1-21.8) culture positive versus culture negative.</td>
</tr>
<tr>
<td>McGregor et al. 1990</td>
<td>double-blind, placebo-controlled trial of short-course erythromycin treatment at 26-30 gw</td>
<td>n=119</td>
<td>treatment unselected gravid women n=119</td>
<td>endocervix culture</td>
<td>PROM (any gestational age) RR 2.5 (95%CI 1.1-5.7, p=0.03) positive versus culture negative placebo versus treatment RR 0.4 (95%CI 0.2-0.8, p=0.01)</td>
</tr>
<tr>
<td>Kovac et al. 1998</td>
<td>prospective, multicenter study</td>
<td>n=6161</td>
<td>endocervix Gen-Probe method</td>
<td></td>
<td>Infection rate 5.74%. No significant correlations between PROM, PTL</td>
</tr>
</tbody>
</table>

gw: weeks of gestation, PTL: preterm labour, PTD: preterm delivery, PROM: premature rupture of the membranes
It has long been recognized that untreated pyelonephritis is associated with PTL. Closer investigation has revealed that even bacteriuria with no clinical symptoms of cystitis or pyelonephritis increases the risk of PTD. Women with untreated asymptomatic bacteriuria have a RR of 1.98 for PTD (Romero et al. 1989a). Antibiotic treatment of asymptomatic bacteriuria reduces this risk by almost 50% based on a meta-analysis of randomized placebo-controlled trials (Romero et al. 1989b).

### 2.4.3 Distant infections and preterm delivery

Infections at sites distant from the uterus can potentially lead to PTD by haematogenous spread of either microbes or cytokines. Periodontal disease is a common chronic inflammatory process involving gram-negative rods and anaerobes (Jeffcoat et al. 2001). Severe or generalized periodontitis has recently been found to be associated with an increased risk of PTD, with an OR of 4.45 (95% CI, 2.16-9.18) after adjustment for other known risk factors (Jeffcoat et al. 2003).

Other infection, such as systemic infections including pyelonephritis, pneumonia and peritonitis, are associated with PTL or PTD, evidently due to the release of microbial endotoxins and other mediators of inflammation (reviewed by Goldenberg et al. 2000).

### 2.4.4 Neonatal consequences of intrauterine infection

The significance of intrauterine infection lies not only in its contribution to the overall problem of preterm delivery, but also in its neonatal sequelae. Several studies have shown how intrauterine infection leads to adverse neonatal outcomes. It has become clear that this foetal response is implicated in both the initiation and the perpetuation of the process of preterm labour and in the subsequent foetal injury (reviewed by Goldenberg et al. 2000). Among patients with PTL or PROM, the presence of a foetal inflammatory response, known as “foetal inflammatory response syndrome” and defined by elevated foetal plasma IL-6, was associated with a fourfold (OR 4.3) risk for severe neonatal morbidity (respiratory distress syndrome sepsis, pneumonia, bronchopulmonary dysplasia, periventricular leukomalacia or necrotising enterocolitis) after correction for gestational age and other variables (Gomez et al. 1998). Elevated amniotic fluid concentrations of tumour necrosis TNF-α, IL-1β and IL-6 correlated with the development of ventricular white matter lesions in preterm infants delivered within 72 hours of amniocentesis (Yoon et al. 1997a). Additionally, studies of the brains of preterm infants who die during neonatal period have shown a strong correlation between the expression of TNF-α, IL-1β and IL-6 in infant brain tissue and periventricular leukomalasia (Yoon et al. 1997b).
2.5 Inflammation and infections in the pathogenesis of preeclampsia and gestational hypertension

2.5.1 Preeclampsia and gestational hypertension

The frequency of preeclampsia ranges between 2% and 7% in healthy nulliparous women (Hauth et al. 2000, Sibai 2005, Vatten & Skjærven 2004). Preeclampsia is characterised by an abnormal vascular response to placentation that is associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system and endothelial cell dysfunction (Roberts et al. 1989, Redman et al. 1999, Report of the National High Blood Pressure Education Program 2000). The maternal factors predisposing to preeclampsia (genetic, constitutional and environmental) and related changes (increased inflammatory markers, dyslipidemia, insulin resistance, endothelial dysfunction and oxidative stress) are also associated with an increased risk of cardiovascular complications later in life (Ramsay et al. 2003, Wilson et al. 2003, Pouta et al. 2004, Haukkamaa et al. 2004). Furthermore, placentas from pregnancies complicated by preeclampsia show atherosis of spiral arterioles, an atherosclerotic-like lesion characterized by foam cell invasion and intravascular fibrin deposition (Roberts et al. 1989).

The clinical findings of preeclampsia can manifest as either a maternal syndrome (hypertension and proteinuria with or without other multisystem abnormalities) characterised by endothelial cell activation (Roberts et al. 1989) or a foetal syndrome (foetal growth restriction, reduced amniotic fluid and abnormal oxygenation) (Redman 1993). Although the etiology of endothelial dysfunction in preeclampsia is mainly unknown, it has been postulated to be part of an exaggerated maternal inflammatory response to pregnancy (Sacks et al. 1998, Redman et al. 1999, von Dadelszen et al. 2003). Key findings support a causal or pathogenetic model of superficial placentation driven by immune maladaptation, with subsequently reduced concentrations of angiogenic growth factors and increased placental debris in maternal circulation, resulting in a (mainly hypertensive) maternal inflammatory response (Sibai et al. 2005).

It has been hypothesised that infection may also be important in the pathogenesis of preeclampsia, both in terms of its initiation (by increasing the risk of uteroplacental atherosis) and/or its potentiation (by amplifying the maternal systemic inflammatory response) (von Dadelszen & Magee 2002). Activated circulating leukocytes (Haeger et al. 1992, Von Dadelszen et al. 1999), increased production of reactive oxygen species (Walsh 1998) and increased release of inflammatory cytokines (Sacks et al. 1998, Williams et al. 1999), such as TNF-α or IL6, and elevated CRP (Qiu et al. 2004) as well as abnormal activation of the clotting system in women with preeclampsia compared with normotensive women support this hypothesis.

Normal pregnancy itself stimulates the maternal inflammatory response, and these changes are exaggerated in preeclampsia. The corollary is that any factor that would enhance this response would predispose to preeclampsia (Redman & Sargent 2003). Recent studies indicate that maternal infections, e.g. cytomegalovirus-related chronic vilitis, chronic gastrointestinal infection, urinary and/or lower genital tract infection, can be associated with preeclampsia (reviewed by von Dadelszen & Magee 2002). The presence of periodontal disease at < 26 weeks’ gestation also increased the risk for subsequent
preeclampsia (Bogges et al. 2003). Periodontal disease, which causes chronic inflammation, has similarly been linked with atherosclerosis (Mattila 1993, Beck et al. 1999). It is obvious that genetic, immunologic and environmental factors are responsible for the development of preeclampsia. It could be clinically useful to distinguish between late and early (before 34 weeks of gestation) onset of preeclampsia because the latter increases both the risk of recurrence in future pregnancies and cardiovascular morbidity (Sibai et al. 2005).

Preeclampsia and gestational hypertension are often considered a continuum of the same disease, the main difference between the two manifestations being that endothelial dysfunction is present in preeclampsia but not equally evident in gestational hypertension (Powers et al. 2001). Although preeclampsia is widely recognized as a leading cause of maternal and fetal morbidity and mortality, gestational hypertension is often considered a benign condition. However, although maternal end-organ damage is more common in preeclampsia, gestational hypertension is also associated with increased rates of caesarean section, preterm delivery and small-for-gestational-age babies (Wolf et al. 2002).

### 2.5.2 Role of CRP

A slightly elevated CRP level, measured by highly sensitive methods (hsCRP), as a marker of systemic low-grade inflammation has been shown in several studies to be a risk factor for or to associate with cardiovascular diseases (Ridker et al. 2000). Three recent studies have reported elevated CRP levels in preeclampsia, (Wolf et al. 2001, Tjoa et al. 2003, Qiu et al. 2004), but other studies have failed to confirm this (Djurovic et al. 2002, Savvidou et al. 2002, Wolf 2002). Vickers et al. (2003) found no difference in CRP levels between patients with either preeclampsia or gestational hypertension and controls; however, women with prior preeclampsia had higher CRP values than those with gestational hypertension.

### 2.5.3 Role of *C. pneumoniae* infections

*C. pneumoniae* infection has been shown to lead to impaired endothelial function in mice and pigs (Ljuba et al. 2000, 2003). In humans, too, a pathogen burden – i.e. the presence of antibodies against several infectious agents – has been shown to be a significant risk factor for endothelial dysfunction (Prasad et al. 2002).

*C. pneumoniae* infection has been linked with essential hypertension (Leinonen & Saikku 2002), and a few reports on the association of *C. pneumoniae* antibodies with preeclampsia have recently also been published. A causal link between *C. pneumoniae* infection and preeclampsia has been suggested. However, the data are very scarce, and the results have been contradictory (Heine et al. 2003, Goulis et al. 2005, Teran et al. 2003, Raynor et al. 2004). The studies on the association between *C. pneumoniae* (*Cpn*) antibodies and CRP in preeclampsia and gestational hypertension are summarized in Table 5.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Study design</th>
<th>Sample size</th>
<th>Gestational week</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Dadelzen et al. 2003</td>
<td>Nested case-control study</td>
<td>n=9 early onset PE, n=29 late onset PE, n=33 IUGR</td>
<td>n=113 normotensive first (no exact gestational weeks reported)</td>
<td>Cpn antibodies higher in early-onset PE than in normal pregnancy and in late-onset PE, no difference between late-onset PE and normal pregnancy (p=0.133)</td>
</tr>
<tr>
<td>Heine et al. 2003</td>
<td>Cross-sectional study</td>
<td>n=37 PE n=37 normotensive third trimester, at the time of admission for labour and delivery</td>
<td></td>
<td>IgG Cpn antibodies more common in PE (25 of 37) than in normal pregnancy (15 of 37), p&lt;0.05 the risk of PE OR 3.7 (95% CI 1.2-7.9)</td>
</tr>
<tr>
<td>Tera et al. 2003</td>
<td>Prospective study</td>
<td>n=84 healthy pregnant women at 16 weeks' pregnancy</td>
<td>n=11 PE n=73 normal pregnancy second trimester (16 gw)</td>
<td>Previous Cpn infection elevated the risk of PE OR 1.8 (95% CI 0.4-9.1)</td>
</tr>
<tr>
<td>Qiu et al. 2004</td>
<td>Prospective nested case-control study</td>
<td>n = 60 PE n=506 normotensive first (13 gw)</td>
<td></td>
<td>CRP associated with PE in lean women OR 2.5 (95% CI 1.1-5.5)</td>
</tr>
<tr>
<td>Tjoa et al. 2002</td>
<td>Prospective study</td>
<td>n=6 PE, n=9 IUGR n=107 low risk women first (10–14 gw)</td>
<td></td>
<td>CRP higher in the PE group than in controls 1.58 vs. 0.67 mg/L, p=0.002 after matching for parity, maternal age and gestational age at delivery p=0.041</td>
</tr>
<tr>
<td>Wolf et al. 2001</td>
<td>Prospective nested case-control study</td>
<td>n=40 PE n=80 normotensive first (11 gw)</td>
<td></td>
<td>CRP higher in the PE group than in normotensive controls 4.6 vs. 2.3 mg/L, p=0.04</td>
</tr>
</tbody>
</table>

Table 5. Studies on C. pneumoniae (Cpn) antibodies and CRP in preeclampsia (PE) and Gestational Hypertension (GH).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Design</th>
<th>Sample Size</th>
<th>Trimester (gestational week)</th>
<th>Outcome</th>
<th>CRP and gestational hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Djurovic et al.</td>
<td>Prospective nested case-control study</td>
<td>n=71 PE, n=71 normotensive</td>
<td>Second (18 gw)</td>
<td>No difference in CRP between PE and normotensive controls (median 4.47 range 0.56–34.20) vs. (median 4.00 range 0.15–27.05, p=0.73)</td>
<td></td>
</tr>
<tr>
<td>Savvidou et al.</td>
<td>Cross-sectional study</td>
<td>n=45 PE, n=45 normal uterine artery Doppler</td>
<td>Second (23–25 gw)</td>
<td>No difference in CRP between cases and controls (median 1.56 range 0.55–3.12) vs. (median 1.28 range 0.75–2.0, p=0.95)</td>
<td></td>
</tr>
<tr>
<td>Belo et al.</td>
<td>Cross-sectional study</td>
<td>n=51 PE, n=67 normotensive</td>
<td>Third CRP higher in PE than in normotensive controls 4.83 vs. 3.18 mg/l, p&lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Teran et al.</td>
<td>Prospective study</td>
<td>n=25 PE, n=26 normotensive</td>
<td>Third</td>
<td>CRP higher in PE than in normotensive controls 4.11 vs. 2.49 mg/l, p=0.001</td>
<td></td>
</tr>
<tr>
<td>Vickers et al.</td>
<td>Prospective study</td>
<td>n=394 PE, n=163 matched controls</td>
<td></td>
<td>No difference in CRP between PE and normotensive controls. Women with prior PE had higher CRP values than those with GH median log 0.70 vs. 0.34, p=0.16</td>
<td></td>
</tr>
<tr>
<td>Wolf et al.</td>
<td>Nested case-control study</td>
<td>n=51 GH, n=102 randomly selected normotensive controls</td>
<td>First trimester (10–12 gw)</td>
<td>No difference in CRP between GH and normotensive controls median 1.8 vs. 2.3 mg/l, p=NS</td>
<td></td>
</tr>
</tbody>
</table>
3 Aims of the study

A unique feature of chlamydial reproductive tract infections is the lack of identifiable clinical symptoms in the majority of infected women and in about half of infected men. The presence of chlamydial antibodies may be the only marker of persistent chlamydial infection, which maintains low-grade inflammation in the target tissue and may gradually lead to severe sequelae. At present, there is a lack of information on the possible association between chronic chlamydial infection, systemic inflammation and reproductive health in the general population. Because chlamydial heat shock proteins, cHsp10 and cHsp60, have been suggested to contribute to the pathogenesis of chronic chlamydial infections, humoral and cell-mediated immunity to cHsp10 and cHsp60 might also play a role in pregnancy complications.

The hypotheses were that 1) previous or chronic *C. trachomatis* infection is associated with subfertility both in females and in males, 2) chronic *C. trachomatis* infection and low-grade systemic inflammation during the first trimester are associated with preterm delivery, and 3) chronic *C. pneumoniae* infection and low-grade systemic inflammation during the first trimester are associated with preeclampsia and gestational hypertension.

The specific aims of the study were:

1. To find out whether there is a serological association between previous *C. trachomatis* infection and subfertility.
2. To study if serum antibodies to cHsp60 and cHsp10 are associated with subfertility.
3. To study whether the antibodies to *C. trachomatis*, chlamydial Hsp60 and Hsp10 and *C. pneumoniae* and hsCRP levels in maternal serum are already elevated during the first trimester in women with preterm delivery.
4. To study whether *C. pneumoniae* infection and inflammation are associated with preeclampsia and gestational hypertension by measuring *C. pneumoniae* antibodies and hsCRP levels in maternal serum obtained during the first trimester.
4 Materials and methods

4.1 Study population

The Northern Finland Birth Cohort 1966 (NFBC 1966) consists of 12 058 live births covering 96.3% of all births in the area with the expected date in the year 1966 (Rantakallio 1969). The mothers were recruited through maternity welfare clinics. Interviews and postal questionnaire were completed from the 24th gestational week onwards (data since 16th gestational week). The children were followed up prospectively. In the last follow-up study in 1998, at the age of 31 years, questionnaires were sent to all traced subjects who were alive, numbering 11 637 (5608 females and 6029 males), and 8690 responded (75%). The postal inquiry included questions on socioeconomic position, education, smoking and reproductive history and data on previous chlamydial infections. An invitation for a clinical examination was sent to those living in the original target area or the capital city area, and 3127 women and 2880 men attended, gave a blood sample and written consent (n=6007, 70% of those eligible). The subjects are representatives of the whole cohort in terms of their social background in early childhood (Järvelin et al. 2004). The following studies are based on this population.

Study design. A nested case-control design was used in a longitudinal population-based birth cohort study. The cases and the controls were selected based on the outcome interest.

Blood sampling. The serum samples were collected from the enrolled women and men in 1997–1998 at the age of 31 years (studies I–II). The serum samples were stored at –20°C until analysed in 2000–2002.

The first-trimester (mean 10.4 gestational weeks) serum samples during the first pregnancy from the enrolled women at a median age of 25 years were obtained from the serum bank of the Finnish Maternity Cohort (FMC) in the National Public Health Institute and were available for 98.5% of the study population. Such samples have been collected and stored (at -25°C) from all (98.5%) pregnant women in Finland since 1983.
4.1.1 Population in the studies on subfertility (I, II)

In both studies, subfertility as outcome was estimated as a time to pregnancy (TTP) of 12 months or longer, which is widely used as the criterion of subfertility (Joffe et al. 1993, 1995). The TTP variable is presented in more detail in table 1 in study I. The studies I and II are based on 4158 subjects for whom data on TTP of the first pregnancy were available and 414 subjects who had currently been trying to conceive having no earlier pregnancies. Those who had used contraception at the time of conception were excluded (n=705).

The studies I and II included altogether 751 subjects with TTP > 12 months, and 493 (304 females and 189 males) of them gave blood samples. Two randomised controls for each case (n=986) were enrolled from those who had had TTP <12 months before the conception of their first pregnancy, had not used contraception at the time of conception, had not felt infertility to be a problem, and had not been examined or treated because of infertility. In study I, the final analysis consisted of 479 cases (298 female and 181 male partners of subfertile couple) and 967 controls (600 female and 367 male partners of fertile couple).

In study II, we further measured *C. trachomatis* Hsp antibodies for the subjects who were EIA-positive in study I and for an equal number of randomly selected EIA-negative control subjects (II, Figure 1). In study II, the final study population consisted of 146 cases (94 females and 52 males) and 278 controls (188 females and 90 males).

4.1.2 Population in the preterm delivery study (III)

The study population of study III was based on the female cohort, derived also from the NFBC66. Altogether 2309 women with a history of at least one delivery and data on their first pregnancy (obtained from the Finnish Medical Birth Register, FMBR) were eligible for the study. The Finnish Medical Birth Register covers all deliveries in Finland from 1987 onwards, including prospective information on pregnancies. Gestational age was determined by menstrual history and confirmed by second trimester ultrasonographic measurement for 75% of the participants. Multifetal pregnancies were excluded. The final cases consisted of 104 women with spontaneous preterm (< 37 gestational week) deliveries and first-trimester serum samples. A fourfold number of age- and parity-matched controls were randomly selected from the same female cohort (n=402, term delivery at or after 37 completed weeks of gestation).

4.1.3 Population in preeclampsia and gestational hypertension study (IV)

The study population of study IV was based on the same female cohort as that of study III. In addition, the women from NFBC66 who returned the postal questionnaire at the
age of 31 years and delivered before the year 1987 (before the FMBR took into use) or
after the clinical examination (1998–2001) were also included in the study.

Data on the first birth were available from 3439 women. The data on the females’ own
births and characteristics were traced from multiple inter-related sources: by the postal
questionnaires and clinical examinations, the Finnish Medical Birth Register (FMBR), the
National Hospital Discharge Register (HDR), hospital charts and the existing NFBC data-
base. First-degree family history of hypertension (i.e. mother’s gestational hypertension/
preeclampsia during pregnancy in 1966) was verified from the NFBC database. The HDR
and FMBR were cross-checked to trace all the women with gestational hypertension with
and without proteinuria for a further hospital chart review of all hypertensive cases.

The first-trimester maternal serum samples for the enrolled women were available for
161 women with gestational hypertension or preeclampsia and for 3219 women with normotensive pregnancy. However, due to the lack of resources, the first-trimester serum
samples were studied randomly from 1479 women with normotensive pregnancy. The
final study population consisted of 77 women with preeclampsia, 84 with gestational
hypertension and 1479 with normotensive pregnancy.

4.2 Laboratory methods

4.2.1 Measurement of C. trachomatis antibodies

4.2.1.1 Microimmunofluorescence test (MIF) (Studies I–III)

C. trachomatis IgG, IgA (Studies I–III) and IgM (Study III) antibodies were measured
with the MIF method using EBs of the C. trachomatis pooled serovars GFK (intermedi-
ate complex), BED (B-complex) and CJHI (C-complex) as antigens (Washington
Research Foundation, Seattle, WA, USA). All C. trachomatis-positive findings were fur-
ther analyzed for antibodies against the serotypes C, J, H, I, F and K obtained from the
Washington Research Foundation (Washington Research Foundation, Seattle, WA, USA)
and G, B, E and D from ATCC (American Type Culture Collection, Rockville, Maryland,
USA). All serotype-specific antigens were tested and diluted before pooling. The reactivi-
ty of the antigen pools was checked by commercial C. trachomatis monoclonal antibody
(Bio-Rad, Redmond WA, USA), which identifies all serotypes. The specificity of the
antigen pools was tested with known positive sera from previous studies. The positive
control sera with known antibody titers were included in every test series, and they
should give the same titer in all titrations. One person prepared the antigen slides with
uniform antigen densities and quality for all experiments. The interpretation of the results
was done by one experienced researcher (M.P.) using a single microscope.

If any antibodies against the serovar pool were present, all C. trachomatis immuno-
types of the pool were further analysed. Fluorescein-conjugated anti-human IgG, IgA
(Kallestad Diagnostic, Chaska, MN, USA) and IgM (Dako, Glostrup, Denmark) were
used as secondary antibodies. The serum samples were analysed at twofold dilutions for
C. trachomatis antigens. Titres $\geq 8$ were considered as positive for C. trachomatis IgG
antibodies, ≥ 8 for IgA antibodies and ≥ 8 for IgM) antibodies. The cut-off titres were chosen based on in-house research experience. In the MIF and other serological tests, the antibody determinations of each case and individual controls were always tested simultaneously in the same titration series in a blinded fashion.

4.2.1.2 Enzyme immunoassays (EIA) (Studies I–II)
We used a peptide EIA as the screening method for *C. trachomatis* antibodies. Total IgG (I–II) and IgA (I–II) antibodies to *C. trachomatis* were analysed according to the manufacturer’s instructions using the EIA test (Labsystems, Finland) and by utilising synthetic peptides from the variable MOMP domain IV of the *C. trachomatis* serotypes C, G, E and L2 as antigen. The result was interpreted to be positive if the signal per cut-off value was ≥ 1 or negative if it was <1. We used lower cut-off values (1.0) for positivity than those recommended (≥ 1.4) by the manufacturer in order also to include cases with lower antibody levels.

4.2.2 Measurement of heat shock protein (Hsp60 and Hsp10) antibodies (Studies II, III)
IgG and IgA antibodies to *C. trachomatis* Hsp60 and Hsp10 were measured with an EIA test developed in our laboratory. Recombinant chlamydial Hsp60 and Hsp10 were kindly provided by Dr. GI. Byrne (Memphis, Tennessee) and purified as previously described (LaVerda & Byrne 1997, LaVerda et al. 2000). Microtitre plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg/ml of the Hsp60 or Hsp10 antigens, 100 μl/well, in PBS and incubated overnight at +37 ºC. After coating, the plates were washed four times with PBS-0.05% Tween 20 by using a SkanWasher (Skatron Instruments As, Norway) plate washer. The sera were diluted 1:200 with PBS containing 10% fetal bovine serum (PBS-FBS) for measurement of IgG and 1:50 for measurement of IgA antibodies and incubated for 120 min at +37 ºC. After washing, alkaline phosphatase-conjugated goat anti-human IgG or IgA (Sigma Chemicals Co., St.Louis, Mo.) diluted 1:1000 with PBS-FBS for measurement of IgG and 1:50 for measurement of IgA antibodies and incubated for 120 min at +37 ºC. After washing, alkaline phosphatase-conjugated goat anti-human IgG or IgA (Sigma Chemicals Co., St.Louis, Mo.) diluted 1:1000 with PBS-FBS was added and incubated for 120 min at +37 ºC. The plates were washed three times with PBS-FBS and two times with distilled water. After adding the substrate, p-nitrophenylphosphate, 1mg/ml, (Sigma FAST tablets, Sigma Chemicals Co., St.Louis, Mo.) in carbonate buffer, pH 9.8, the plates were incubated for 30 min at + 37 ºC. After stopping the reaction with 2 N NaOH, absorbances were measured at 405 nm by a Labsystems Multiskan MCC/340 (Thermolabsystems, Finland) spectrophotometer. For each plate, the absorbance value of the blank (a coated well with dilution buffer instead of patient serum) was subtracted from the values of all test wells. The results were expressed as EIA units (optical density value x serum dilution).
The EIA unit values above medians were considered as elevated. The median (range) of IgA and IgG antibodies to *C. trachomatis* Hsp60 and Hsp10 in cases and controls were calculated separately for females and males.

### 4.2.3 Measurement of *C. pneumoniae* antibodies (Studies I–IV)

*C. pneumoniae*-specific IgG (I–IV), IgA (II–IV) and IgM (III–IV) antibodies were measured with an in-house MIF test using purified elementary bodies (EB) of the Finnish strain Kajaani 6 (I–IV) as antigen and fluorescein-conjugated anti-human IgG, IgA and IgM (Dako, Glostrup, Denmark) as conjugates. The serum samples were analysed at fourfold dilutions for *C. pneumoniae* antibodies. In IgA and IgM antibodies, false positive reactions were avoided by using Gullsorb (Gull Laboratories, Salt Lake City, Utah) reagent. IgG titers of $\geq 32$ and IgA and IgM titers of $\geq 10$ were defined as positive for *C. pneumoniae* antibodies.

### 4.2.4 Measurement of hsCRP (Studies III, IV)

The serum hsCRP concentrations reported in the studies III and IV were measured with a commercial highly sensitive immunoenzymometric assay (IEMA) kit according to the instructions (Medix Biochemica, Kauniainen, Finland). The enzymatic reaction was proportional to the amount of hsCRP in the sample. The sensitivity of the test was 0.08 mg/l and the assay range from 0.3 to 30.0 mg/l.

### 4.2.5 Measurement of serum cotinine (Study IV)

Serum cotinine levels were measured by a competitive micro-plate immunoassay (OraSure Technologies, Inc., Bethlehem, PA) and used as a surrogate marker for smoking. Cotinine levels were measured, because smoking might be a confounding factor when analysing the association between *C. pneumoniae* antibodies and preeclampsia. A cut-off level of 20 ng/ml was used to distinguish active smokers from nonsmokers (Parish et al. 1995).

### 4.3 Statistical power and analyses

Power calculations were based on known occurrences of *C. trachomatis* or *C. pneumoniae* antibody prevalence in the general population and on adequate differences in occurrence between cases and controls in each study separately. In study III, for example, a
pre-study calculation indicated that if a 20% occurrence of *C. trachomatis* antibodies in the general female population is assumed, this study has 85% power to detect a 15% difference between the cases and the controls at a 5% significance level.

Categorical variables were compared by Chi-squared test or Fisher's exact test as appropriate (studies I, II, III and IV). Student’s t-test was used to compare age and body mass index (studies III, IV) and birth weight (study IV). The non-parametric Mann-Whitney U-test was used to compare the continuous variables (studies I, II, III, IV) between the study groups due to the originally skewed distributions. Spearman's correlation coefficient was used for an analysis of correlation (studies I, II, III). For study I, antibody titres were transformed to natural logarithms for the calculation of their geometric means (GMT) and 95% confidence intervals (95% CI). In study II, Hsp60 and Hsp10 antibodies were categorised by upper quartiles separately for females and males.

When the joint effect of *C. trachomatis* IgG antibodies and hsCRP levels was studied in study III, a new categorical variable was constructed to represent the different combinations of the original variables. To estimate the relative risk of preterm delivery associated with these combinations, odds ratios with 95% confidence intervals were calculated using logistic regression analysis.

Statistical analyses were performed with SPSS software for Windows (versions 9.0 and 11.5, SPSS Inc. Chicago, USA).

### 4.4 Ethical considerations

Informed consent was obtained from all participants. Permissions and ethical approval for the studies have been given by the Ministry of Health and Social Affairs and the Ethics Committee of the University of Oulu and for the use of maternal serum samples by the FMC steering group in National Public Health Institute.
5 Results

The detailed results are presented in the original publications and are only summarized here briefly.

5.1 Association between *C. trachomatis* antibodies and subfertility (Study I)

In our original study population (n=8690), 8% of the males and 10.5% of the females reported subfertility (TTP ≥12 months) in their couple relationship.

Although cases had serum EIA IgG antibodies to *C. trachomatis* present more often and in higher titres than controls, no statistically significant difference was found.

However, serum *C. trachomatis* immunotype-specific antibodies were associated with subfertility both in men and in women. In the analysis of immunotype-specific *C. trachomatis* antibodies, the most distinct differences between cases and controls were seen in the antibodies against the C-complex consisting of the immunotypes C, J, H and I. 7.7% of the sera from the male cases and 3.0% of the sera from the male controls showed IgG antibody specificity for pooled CJHI serotypes (p = 0.012), and the corresponding figures among the women were 8.1% and 5.5% (p = 0.139). Among the single *C. trachomatis* serotypes, the frequency of IgG antibodies was generally higher in the cases than in the controls, being statistically significant for serotype H among males and for the serotypes C, J, H and I among females. (I, Table 4).

*C. trachomatis* antibodies were more often present in males with self-reported, verified prior chlamydial diagnosis compared to those without a reported history of *C. trachomatis* infection (in cases 17.6% and 6.7%, in controls 12.2% and 4.9%, respectively). The same phenomenon was also seen in females (in cases 21.1% and 8.1%, in controls 23.5% and 7.3%). The difference was not statistically significant between the cases and the controls.
5.2 Association between antibodies to *C. trachomatis* Hsp60 and Hsp10 and subfertility (Study II)

Among the female partners of subfertile partnerships, the medians of IgA antibodies to *C. trachomatis* Hsp60 and Hsp10 were significantly higher in the cases than in the controls: the median for IgA Hsp60 antibody levels was 30.4 for the cases vs. 25.3 for the controls (p=0.002), and the medians of IgA Hsp10 antibody levels 29.5 for the cases vs. 25.9 for the controls (p=0.007). The medians of IgG antibodies to *C. trachomatis* Hsp60 and Hsp10 antibodies were generally higher in the cases than in the controls, but the serological association was weaker and not statistically significant. After stratification by smoking status, due its possible confounding effect, the results showed a similar tendency, though the association was weaker.

Among the male partners of subfertile couples, the median of IgA antibodies to Hsp60 and Hsp10 (28.7 and 25.9) were lower compared to the male controls (33.0 and 30.1, p values 0.099 and 0.048, respectively). The IgG antibody levels to Hsp60 were lower in the cases than in the controls and IgG antibody levels to Hsp10 were higher in the cases than in the controls, but the differences were not statistically significant. Among the male smokers, the levels of IgA antibodies to *C. trachomatis* Hsp60 and Hsp10 were lower in the cases than in the controls, but the serological association was not statistically significant. Among the male smokers, the medians of IgG antibodies to Hsp60 antibodies were lower in the cases than in the controls (36.9 and 43.8, p values 0.042, respectively) (II, Table 1).

The percentages of subjects with antibody levels above the upper quartile were higher among the female cases than their controls, the difference being statistically significant only for Hsp10 IgA (34.4% vs. 20.7%, p=0.013). Antibodies to both Hsp60 and Hsp10 were more common in the female partners of subfertile couples than in their fertile controls, the difference being statistically marginal (p value for IgA 0.053 and for IgG 0.091).

5.3 Association of *C. trachomatis* antibodies and hsCRP levels with preterm delivery (Study III)

The hsCRP levels (mg/l) in the sera collected in early pregnancy were significantly higher in the women with preterm delivery than in those with term delivery (median 2.7 [interquartile range, 1.1–5.8] vs. 2.0 [0.7–4.1], p = 0.007). The percentage of subjects at or above the upper quartile (4.3 mg/l) was also higher among the cases than the controls (34.6% vs. 22.4%, p = 0.010). IgG antibodies to *C. trachomatis* serovar pools and to individual serotypes were more often present in the first trimester sera of the women with preterm delivery compared to those delivered at term (any pool positive 28.8% vs. 20.6%, p = 0.074), the difference being nominally significant only for serotype I.

The presence of *C. trachomatis* antibodies alone without elevated hsCRP did not increase the estimated risk (OR=1.0, 95% CI 0.5–2.0), and when elevated hsCRP was present without *C. trachomatis* antibodies, the estimated risk did not increase significantly (OR=1.3, 95% CI 0.7–2.3). The simultaneous presence of these factors increased
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the estimated risk 4.3-fold (95% CI 2.0–9.3). Among the cases with delivery at or before 34 weeks of gestation, the estimated risk was even more evident (OR 5.6, 95% CI 2.1–14.5) (III, Table 3).

5.4 Association of *C. pneumoniae* antibodies and hsCRP levels with preeclampsia and gestational hypertension (Study IV)

There were no significant differences in the seroprevalence rates or in the levels of *C. pneumoniae* IgG antibodies and the hsCRP levels between the women with preeclampsia or gestational hypertension and the reference group.

Our results showed that preeclamptic women with preterm delivery had significantly more often serum IgG antibodies to *C. pneumoniae* (IgG titer ≥ 32) in their first trimester sera compared with the preeclampsia group delivered at term (43.8% vs. 15.5%, *p* = 0.034). No significant differences in the hsCRP levels were seen between the preeclamptic women who delivered preterm and those with term delivery.

However, in the preeclamptic group, the proportions of subjects with *C. pneumoniae* IgG antibodies (IgG titer > 32) and/or elevated hsCRP levels (3.8 mg/l, upper quartile) were significantly higher among the women delivered preterm compared to the women delivered at term (68.8% vs. 34.5%, *p* = 0.014, in the study IV, Table 3). In the normotensive reference group, there was no significant difference in the proportions of subjects with *C. pneumoniae* IgG antibodies (IgG titer 32) and/or elevated hsCRP levels (3.8 mg/l, upper quartile) in the first trimester sera between the women with preterm delivery and the women delivered at term (54.2% vs. 45.6%, *p* = 0.127).
6 Discussion

6.1 Study population

The major strengths of the present study are the data sources. The study is based on a birth cohort representing the unselected general population. The birth cohort included 96% of all live births (n=12 058) in the provinces of Lapland and Oulu in 1966. Data on the biological, socioeconomic and health conditions of the cohort members have been collected prospectively since the fetal period (Rantakallio 1988). This makes an analysis of intervening and confounding factors possible. The study population is well defined and consists exclusively of Caucasians, suggesting that selection bias is unlikely. The subjects at age 31 were representative of the whole cohort in terms of their social background in early childhood (Järvelin et al. 2004). Generally, evidence based on longitudinal studies provides more powerful causal inferences than evidence based on cross-sectional studies.

The studies on subfertility (I–II) were nested case–control studies, and the controls were matched for age and sex. This is important since the characteristics of subfertility are strongly age- and sex- dependent. This unique study population provided us with the possibility to evaluate the role of C. trachomatis infection in the general population and in the early phase of fertility problems. Earlier studies have mostly been hospital-based case-control studies on relatively small samples.

The study on preterm delivery (III) was also a nested case–control study, and the subjects and their controls were nulliparous. The data on cohort members’ pregnancies were collected from the Finnish Medical Birth Register (FMBR), which covers all deliveries in Finland from 1987 onwards and includes prospective information on pregnancies.

The study on preeclampsia and gestational hypertension (IV) had the same design as study III, with the population including only nulliparous women. The data on hospital-treated gestational hypertension or preeclampsia in the cohort members were collected from the Finnish Medical Birth Register (FMBR) and the national Hospital Discharge Register (HDR) covering all Finnish hospitals.

The serum bank of the Finnish Maternity Cohort (FMC) in the National Public Health Institute and FMBR are ideal for systematic evaluation of exposure to chlamydial infections and identification of individuals who reach the endpoint (III, IV). First-trimester
serum samples were available for 98.5% of the study population. Obstetric cases and their controls were identified by linking the data files of the serum bank and the FMBR using unique personal identification codes.

6.2 Methods

*Chlamydia trachomatis* infection of the upper genital tract can exist in women without *C. trachomatis* isolation from the lower genital tract. Such infection can persist undetected without any symptoms or signs (Rahm et al. 1988). In this kind of a situation, the presence of antibodies against the infecting microbe might be the only indicator of persistent infection. In this study, we used MIF (I–III) and EIA (I) for the measurement of *C. trachomatis* antibodies, and MIF was also used for the measurement of *C. pneumoniae* antibodies (I–IV).

The MIF test developed by Wang and Grayston (1970) is considered the golden standard for the serological diagnosis of chlamydial infections. When performed and interpreted properly, it is a specific method detecting antibodies to chlamydial surface proteins. In the analysis of chlamydial antibodies, the lack of reliable markers for chronic infection and the large variation of methodology (Dowell et al. 2001) are weaknesses, because the results are dependent on the method and the antigen used. Thus, it is essentially important that the samples of cases and controls are analysed simultaneously and blindly by the same person. In our study, the antigen slides for MIF were prepared on the preceding or the same day when the serum dilutions were done, and negative and positive controls were included in every test run. The slides were prepared by experienced laboratory technicians, who maintained the same antigen density and quality in all test runs. Correct interpretation of slides was confirmed by an experienced researcher (M. Paldanius, one of the co-authors in studies I–IV) using a high-quality microscope. Peeling and her colleagues (2000) did pioneering work to improve the international standardisation of MIF, but marked variation in antibody titres between different laboratories still exist.

EIA tests have been developed to meet the need for rapid, technically less demanding and reliable serological tests for the detection of chlamydial antibodies. EIA tests, although described as early as the 1980s, were introduced relatively late into clinical practice due to the problems in preparing species-specific antigens. The objectivity of the results and the automation of the laboratory processes are the strengths of EIAs, but the specificity of the tests and the lack of validation are weaknesses. In addition, EIA tests have been developed for the diagnosis of acute infections, and their applicability to chronic infections has not been studied properly. In the present study, we used commercial peptide EIA, utilizing synthetic peptides from the variable MOMP domain IV of the *C. trachomatis* serotypes C, G, E and L2 as the screening method for the presence of *C. trachomatis* antibodies. This method has been shown to be highly sensitive in the measurement of *C. trachomatis* antibodies (Clad et al. 2000). So far, only a very limited number of studies comparing MIF and EIA have been done. No studies are available in which chlamydial antibody titres measured by EIA and MIF have been compared in predicting tubal factor subfertility. Although chlamydial antibody assays have been incorporated in
the fertility work-up on a large scale, there is no uniformity concerning the assays and antibodies used, and appropriate cut-off levels for titres and definitions of tubal pathology are lacking.

The EIA methods used in the measurement of antibodies to chlamydial Hsp10 and Hsp60 were developed in the Laboratory for Chlamydia and Respiratory Tract Bacteria at National Public Health Institute. Our laboratory has earlier used similar EIA methods for the measurement of IgG and IgA antibodies to human and *C. pneumoniae* Hsp60 proteins. Based on the results, elevated antibodies to *C. pneumoniae* Hsp60 are associated with asthma (Huittinen et al. 2001), and the simultaneous presence of IgA antibodies to *C. pneumoniae* and human Hsp60 is a significant risk factor for cardiac events (Huittinen et al. 2002). Thus, the EIA methods used for the measurement of antibodies against *C. trachomatis* Hsp10 and Hsp60 antigens could be considered reliable. Furthermore, the Hsp10 and Hsp60 preparations used in the present study we obtained from Dr. G. Byrne (Wiskonsin), and similar EIA methods with the same Hsp antigens have recently been used successfully by LaVerda et al. (2000) in assessing the severity of genital tract disease.

### 6.3 Major findings

#### 6.3.1 C. trachomatis infection and subfertility

Antibodies to *C. trachomatis* and female fertility (I). Tuboperitoneal factors (post-infectious tubal damage, tubal obstruction and pelvic adhesions) are the main cause of subfertility in 10–30% of cases in developed countries (Collins 1995). Seropidemiological studies have indicated that chlamydial infections account for a large proportion of these asymptomatic infections by demonstrating a strong link between the presence of serum antibodies to *C. trachomatis* and tubal pathology: the majority of tubal pathology cases are due to chlamydial infections, and the development of late sequelae is associated with the presence of IgG antibodies to *C. trachomatis* (Punnonen et al. 1979).

Our finding showed an association between serotype-specific *C. trachomatis* antibodies measured by MIF and subfertility in women. The differences in *C. trachomatis* antibodies between the cases and controls could be attributed to the C-complex and, within it, to the single serotypes C, J, H and I in females and serotype H in males. There is no conclusive evidence to suggest that specific genital syndromes or clinical manifestations, such as PID, are specifically linked to certain serotypes (Paavonen & Eggert-Kruse 1999). However, Dean et al. (2000) have shown that almost all patients with recurrent *C. trachomatis* infection are infected with uncommon serotypes of the C-complex, suggesting that the C-complex is associated with chronic or recurrent infections.

Laparoscopy with dye chromaerturbation is considered the best available test for diagnosing TFI and the accepted reference test in the evaluation of the diagnostic performance of other tests. But, as general anaesthesia is required, laparoscopy is unsuitable for screening purposes on a large scale. After the discovery that the majority of tubal pathology cases are due to chlamydial infections, and the development of late sequelae is associ-
ated with the presence of IgG antibodies to *C. trachomatis* (Punnonen et al. 1979), chlamydial antibody testing of serum has been introduced as a screening method for TFI (Land et al. 1998, Akande et al. 2003).

Although chlamydial antibody testing with MIF has been incorporated in the fertility work-up on a large scale, no uniformity exists in the assays and appropriate cut-off levels for titres, and definitions of tubal pathology are lacking. A meta-analysis (Mol et al. 1997) showed that the sensitivity of MIF antibody testing is 30–88% and specificity 45–100%. To find reasons for the rather inconsistent and generally poor results, the methodological aspects of antibody testing have been investigated. The predictive value of antibodies depends on the definition of TFI. It has been shown that antibody testing is most accurate in predicting distal tubal pathology instead of unspecific tuboperitoneal abnormalities or proximal tubal occlusion (Land et al. 1998). Further, predictability depends on the cut-off titre for a positive test. It has been noted that higher cut-off titres will improve specificity at the expense of sensitivity (Land et al. 1998). According to receiver operator characteristic (ROC) curves, which show the best combination of sensitivity and specificity, Land and co-workers (1998) found the most suitable statistical cut-off titre to be 16. But when establishing a clinically relevant cut-off level for a positive test, the purpose of screening should be taken into consideration as well.

Although it is assumed that chlamydial infections give rise to persistent IgG antibody formation, the exact course of titres over time is still poorly understood. Because most women have *C. trachomatis* infections during adolescence and seek fertility clinics 10–15 years later, IgG antibodies might become undetectable within this period. Several studies have reported on the course of IgG antibodies after antibiotic treatment of chlamydial infections, showing a significant decline of titre in 37% of women over 1–6 years, but seroconversion from positive to negative has been rare (Puolakkainen et al. 1986, Piura et al. 1993, Henry-Suchet et al. 1994). We found only one study concerning asymptomatic, untreated, subfertile women (Gijsen et al. 2002). In patients with initial chlamydia antibody testing, CAT>32, a decline in IgG antibody titres of two or more titre steps was found in 18% during 4–7 years, but in none did IgG titres become undetectable by MIF (Gijsen et al. 2002). Therefore, it can be assumed that chlamydial IgG antibodies persist for a long time, even after antibiotic treatment, and that a decline in the IgG antibody titre is not a significant cause of a false negative antibody finding. In discussing false positive antibody results, i.e. positive findings in patients without tubal pathology at laparoscopy, the possible cross-reactivity in MIF tests between *C. trachomatis* and *C. pneumoniae* (Moss et al. 1993, Land et al. 1998) should be kept in mind, especially if the tests are done in laboratories without sufficient experience of the interpretation of the MIF test.

In the present study, *C. trachomatis* antibodies, especially those revealed by a specific MIF test, were associated with subfertility both in women and in men. Our results are in accordance with the other studies where chlamydial antibody testing has been shown to have predictive value in the detection of infertility due to tubal damage (Gijsen et al. 2002, Land and Evers 2002). Our results suggest that chlamydial antibody testing could also be a useful screening test in the early phases of fertility work-up.

In earlier studies, the study populations have mainly consisted of clients of infertility clinics. This unique study population provided us with the possibility to evaluate the role of *C. trachomatis* infection in the general population and not in the biased population seeking help from infertility clinics. TTP, i.e. the time that elapsed from the time the cou-
ple started trying to conceive to the time the woman became pregnant, is the best-known measure of infertility at the population level, and it is widely used as a criterion of subfertility (Joffe et al. 1993, 1995). TTP has not been applied in this context earlier.

Antibodies to C. trachomatis and male fertility (I). In males, C. trachomatis is a common cause of urethritis. Up to 50% of infected men are asymptomatic (Zelin et al. 1995). Untreated urethritis can be complicated by epididymitis and prostatitis, the complication rate being below <4% (Thomas et al. 1990). Besides spermatozoa being the vehicle for the transmission of this organism to women, it remains unclear whether C. trachomatis influences the fertilizing capacity of spermatozoa and, if so, whether an immune-mediated mechanism is involved. The role of C. trachomatis infection in male infertility has remained controversial, but the present study suggests that it is also important in males. Our findings point to the possibility that untreated and chronic C. trachomatis infections may lead to elevated antibody levels even in males. In males, antibodies against the C-complex and serotype H were significantly more common in the cases than in the controls. These findings suggest that serotype H might be more closely associated with the development of male infertility than other C. trachomatis serotypes.

There is no convincing evidence for a relationship between previous chlamydial infection and semen quality (Paavonen & Egger-Kruse 1999, Eley et al. 2005b). It has been suggested that serum antibodies may not be reliable markers of previous or current exposure to C. trachomatis infection (Pate et al. 2000). However, the presence of chlamydial IgG or IgA antibodies measured with insensitive single-antigen MIF in seminal plasma, suggesting a previous sexually transmitted infection, has been shown to be related to chlamydial IgG antibodies in serum (Eggert-Kruse et al. 1996). In a large study of 1303 partners of infertile couples who were asymptomatic for genital tract infections, IgG antibodies to C. trachomatis were detected in 12.6% of the men. No relationship between chlamydial IgG antibodies in serum and semen quality in subfertile men was found (Eggert-Kruse et al. 1997). However, chlamydial antibodies in semen were related to chlamydial IgG antibodies in the serum of the female partners obtained at the same time. These findings suggest that the main influence of C. trachomatis on male reproduction is based on sexual transmission and a negative effect on the tubal function of the female partner, but not on reduced functional capacity of sperm (Eggert-Kruse et al. 1996, 1997). In addition, in vitro, co-incubation of sperm with C. trachomatis LPS resulted in cellular death partly due to apoptosis (Eley et al. 2005a). These findings provide an explanation as to how C. trachomatis could mediate premature death in human sperm.

Our results confirm the serological association between past C. trachomatis infections and subfertility and suggest a rather high incidence of undiagnosed C. trachomatis infections in the male partners of subfertile couples. Thus, the present results further suggest that serology might be a useful screening method in infertility investigations in men.
6.3.2 Antibodies to *C. trachomatis* heat shock proteins Hsp60 and Hsp10 and subfertility

We demonstrated a serological association between previous *C. trachomatis* infections, humoral immune response to chlamydial Hsps and female subfertility in this population-based study. Thus, our results agree with the earlier clinical studies showing that antibodies to *C. trachomatis* Hsps are more common in infertile women than in controls (Freidank *et al.* 1995, Claman *et al.* 1997, LaVerda *et al.* 2000) and give further evidence for the role of *C. trachomatis* infections in the development of female infertility.

Not every woman with genital chlamydial infection will develop tubal pathology. A number of women with chlamydial IgG antibodies in serum have no tubal pathology at laparoscopy. Only after chronic inflammation following persistent or recurrent infections, may a delayed hypersensitivity reaction be elicited with subsequent tubal scarring (Patton *et al.* 1994). It has been postulated that the *C. trachomatis* heat shock proteins Hsp60 and Hsp10 play a crucial role in this chronic inflammatory process, because antibodies to these proteins are more common in infertile women than their fertile controls (Arno *et al.* 1995, Freidank *et al.* 1995, Claman *et al.* 1997, LaVerda *et al.* 2000). The exact mechanisms whereby Hsps contribute to infertility and problems in reproduction are not known, but several speculative mechanisms have been suggested. Many couples with fertility problems might have had previous genital tract infections and become sensitised to microbial Hsps. During most infections, the synthesis of Hsps is strongly upregulated, leading to an intense immune response. Because there is high amino acid sequence homology between microbial and human Hsps (Jones *et al.* 1993), the induced immune response against microbial Hsps may incite an autoimmune inflammatory reaction in the host. The cells chronically infected with *C. trachomatis* continue to produce both chlamydial and human Hsps at high levels (Beatty *et al.* 1993). Prolonged and asymptomatic infections may trigger an immune response to chlamydial Hsp epitopes, which cross-react with Hsp epitopes in the host cells (Witkin *et al.* 1998), leading to host tissue damage by autoimmune mechanisms. Antibodies to chlamydial Hsps might predict chlamydia-associated tubal pathology even more accurately than *C. trachomatis* IgG antibodies measured by MIF. MIF tests probably identify the patients who have had chlamydial infections, whereas the presence of antibodies against chlamydial Hsps antibody might identify the patients with chronic infection and likely to develop late sequelae.

Our results indicate a serological association between previous *C. trachomatis* infections, the humoral immune response to Hsps and female subfertility in a population-based sample. They further highlight the need for population-based and clinical studies to clarify the role of chlamydial heat shock protein antibodies compared to the presence of *C. trachomatis* MIF antibodies at initial infertility evaluation, and to assess whether it will provide a rapid non-invasive tool for screening subfertility.
6.3.3 C. trachomatis antibodies in preterm delivery

Our main finding was that elevated C. trachomatis IgG levels and elevated hsCRP levels present in the first trimester already were indicative of preterm delivery among nulliparous women. When both elevated C. trachomatis IgG and elevated hsCRP levels were present, the estimated risk for preterm delivery was over 4-fold. These findings point to the possibility that chronic C. trachomatis infection may lead to systemic low-grade inflammation, as indicated by the elevated hsCRP levels, contributing to the pathogenetic process leading to preterm delivery in our study population.

C. trachomatis may persist in the upper genital tract for months or even years (Shepard & Jones 1989), and the persistent infection in the endometrium may cause repeated adverse pregnancy outcomes. A pre-existing subclinical intrauterine inflammatory process in early gestation has been proposed as a possible condition leading to preterm delivery (Wenström et al. 1998, Hvilson et al. 2002). It has been hypothesized that inflammation of decidual tissue or chorioamnion leads to prostaglandin production, cervical ripening and subsequent uterine contractions (Romero et al. 1988). It is not known exactly when this inflammatory process begins, or how long a latency period is required for the symptoms to manifest. Earlier studies have shown that elevated CRP levels (acute-phase response) are associated with chorionamnionitis (Burrus et al. 1995, Yoon et al. 1996).

Recently, it has been suggested that even a very small rise of the CRP level above the baseline may be a useful predictor of low-grade inflammation (Ablij & Meinders 2002). In the present study, we demonstrated that serum hsCRP levels were already higher during the first trimester in the women with preterm deliveries than in the women with term deliveries. There are only two previous prospective studies on the association between maternal serum hsCRP and preterm delivery. Of these, the study of Hvilson et al. (2002) is in accordance with our study indicating high mid-term maternal hsCRP associating with a nearly twofold risk of preterm delivery. However, Ghezzi et al. (2002) could not confirm this finding.

In addition, we found that only C. trachomatis IgG antibodies were associated with preterm delivery. IgA antibodies were very rare both in the cases and in the controls, and no significant differences in their presence could be found between the groups. Interestingly, IgM antibodies were still found, suggesting that IgG seropositivity indicates a past or possibly persistent or reactivated and non-acute C. trachomatis infection. As regards all sexually transmitted infections, the evidence showing C. trachomatis to be a causative agent for preterm birth is the most powerful, and this infection has been reported to associate with prematurity in several studies (Andrews et al. 2000, Goldenberg et al. 2000). C. trachomatis may occur in endometrial tissue without obvious serological responses (Ersbak et al. 1990) or manifest clinical symptoms (Wiesenfeld et al. 2002) and it may also spread to the placenta and the fetus (Gencay et al. 1997, Andrews et al. 2000, Kramer et al. 2000). Harrison et al. (1983) showed that C. trachomatis IgM-positive women had more often low-birth-weight infants and more often experienced premature rupture of membranes than either IgM-negative or C. trachomatis culture-negative women. In addition, Gencay et al. (2000) showed recently that C. trachomatis IgG antibodies as markers of past infection were more often detected in the sera of mothers with stillbirth, while mothers with very preterm delivery (<30 weeks of gestation) had more often serum IgM antibodies as a marker of acute infection compared to those with term delivery. In our
study, 85.6% of preterm deliveries occurred at gestational ages of over 32 weeks, and the absence of IgM antibodies in our cases may thus indicate that chronic *C. trachomatis* infection plays a more important role than acute infection in this group.

The presence of Hsps in different tissues relevant to human reproduction has been demonstrated: human Hsp60 is expressed during the early stages of pregnancy by glandular epithelial cells in the decidua and follicular fluids of women with failed in vitro fertilisation cycles and also in mammalian embryos (Neuer et al. 2000). Previous studies have shown the presence of Hsp in placenta to be similar in both preterm and term pregnancies, indicating that their production is part of the physiological pregnancy process (Diverset et al. 1995, Ziegert et al. 1999). However, immune complexes between IgG antibodies and Hsp60 and Hsp70 were only detected in the placentas of women delivered preterm (Ziegert et al. 1999), suggesting that autoimmunity to Hsp might be involved in immune-mediated preterm labour. In women previously sensitised to cHsp60, the expression of Hsp60 during the early stages of pregnancy may reactivate the cHsp60-sensitised lymphocytes. The resulting activation of the immune system could lead to a failure of the pregnancy to progress (Neuer et al. 2000, Witkin et al. 2002). In addition, Witkin et al. (1996) have shown that a previous infection with *C. trachomatis* and the resulting immune sensitisation to cHsp epitopes were associated with a poor prognosis for reproductive outcome. Interestingly, in the present study, IgG antibody levels against cHsp60 and cHsp10 were lower in maternal first-trimester sera in the mothers with preterm delivery than in those with term delivery, suggesting that the highly cross-reactive serum antibodies, cHsp proteins, might have been bound as immune complexes to placental tissue expressing both human Hsp (hHsp) and cHsp antigens.

The results of the present study suggest that chronic chlamydial infection detected by the presence of antibodies in serum in the first trimester already may be associated with preterm delivery. When elevated *C. trachomatis* IgG levels were present simultaneously with elevated hsCRP, the risk for preterm delivery was significantly increased. Our findings indicate the need for further studies on the pathogenetic mechanisms of chronic infections in this important problem of pregnancy.

### 6.3.4 *C. pneumoniae* antibodies and preeclampsia or gestational hypertension

According to this study, the preeclamptic women who delivered preterm had significantly more often serum IgG antibodies to *C. pneumoniae* during the first trimester of pregnancy compared to those preeclamptic women who delivered at term. Our data confirm the findings of von Dadelszen et al. (2003), who showed in their nested case-control study, that women with early-onset preeclampsia (< 34 weeks of gestation) had higher serum *C. pneumoniae* antibody levels during the second trimester than women with late-onset preeclampsia (≥ 34 weeks of gestation) and normal pregnancy. Heine et al. (2003) also found in their cross-sectional study increased seroprevalence of *C. pneumoniae* IgG antibodies in women with preeclampsia at the time of admission for labour and delivery when compared with normal pregnancy. In addition, a recent study by Goulis et al.
(2005) showed that women with a history of preeclampsia had higher titres of all classes of *C. pneumoniae* antibodies than women with previous normotensive pregnancy. However, in our study as well as in two other recent studies, *C. pneumoniae* antibodies were overall not more common in preeclamptic women than in those with normal pregnancy (Teran et al. 2003, Raynor et al. 2004).

There is good evidence for an enhanced inflammatory response in women with overt preeclampsia (Sacks et al. 1998, Redman et al. 1999). In addition, three recent studies reported elevated CRP levels in patients with preeclampsia (Wolf et al. 2001, Tjoa et al. 2002, Qiu et al. 2004), although three other studies failed to confirm this (Djurovic et al. 2002, Savvidou et al. 2002, Wolff et al. 2002). Vickers et al. (2003) found no difference in CRP levels between patients with either preeclampsia or gestational hypertension and controls; however, women with prior preeclampsia had higher CRP values than those with gestational hypertension. The present prospective study failed to demonstrate any difference in the presence of elevated first-trimester maternal blood hsCRP levels (highest quartile) between women with preeclampsia or gestational hypertension and normotensive pregnant women. However, among preeclamptic women, elevated hsCRP levels were more often present, especially in those who delivered preterm than in those at term.

Interestingly, in the present study, *C. pneumoniae* antibodies were not associated with gestational hypertension, but elevated levels were seen in preeclamptic women with preterm delivery. Given that endothelial dysfunction is the major clinical feature for the differentiation of these two disease entities, *C. pneumoniae* infection has been shown to lead to impaired endothelial function in mice and pigs (Ljuba et al. 2000, 2002), and in humans, the pathogen burden – i.e. the presence of antibodies against several infectious agents – has been shown to be a significant risk factor for endothelial dysfunction (Prasad et al. 2002). The present results suggest that chronic *C. pneumoniae* infection through endothelial dysfunction might be involved in the development more severe preeclampsia, which then leads to preterm birth.
7 Conclusions

This study showed a serological association between immunotype-specific *C. trachomatis* IgG antibodies and subfertility both in men and women. Our results confirm the serological association between past *C. trachomatis* infections and subfertility and suggest a rather high incidence of undiagnosed *C. trachomatis* infections in the male partners of subfertile couples. Thus, the present results further suggest that serology might be a useful screening method in infertility investigations.

The serological association of IgA and IgG antibodies with the Hsp60 and Hsp10 proteins of *C. trachomatis* and female subfertility was also demonstrated in this study. Thus, our results agree with the earlier studies showing that antibodies to *C. trachomatis* Hsps are more common in subfertile and/or infertile women than in their fertile controls and provide further evidence for the role of *C. trachomatis* infections in the development of female infertility. The present results further highlight the need for population-based and clinical studies to clarify the significance of the combination of chlamydial heat shock proteins at the initial infertility evaluation, and to assess whether it provides a rapid non-invasive tool for screening subfertility.

We further showed the association between hsCRP concentrations in first-trimester maternal sera and preterm delivery. We were also able to show that IgG antibodies against *C. trachomatis* were more often present in the mothers with preterm delivery than in those with term delivery. In conclusion, the present results suggest that chronic chlamydial infection present in first trimester already is associated with preterm delivery. When elevated *C. trachomatis* IgG levels were present simultaneously with elevated hsCRP level, the risk for preterm delivery significantly increased. Our findings indicate the need for further studies on the pathogenetic mechanisms of chronic chlamydial infections in this important problem of pregnancy.

In addition, we showed that *C. pneumoniae* IgG antibodies and elevated hsCRP levels were more often present in preeclamptic women with preterm delivery than in preeclamptic women with term delivery. The results point to the possibility that inflammation linked with chronic *C. pneumoniae* infection and, in at least part of the cases, present during the first trimester can be associated with subsequent development of more severe hypertensive disorders, which results in preterm delivery. Further studies are needed to elucidate the role of chronic infections in the pathogenesis of preeclampsia.

In conclusion, a seroepidemiological association between chlamydial infections and subfertility, preterm delivery and preeclampsia was demonstrated in this birth cohort.
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Liisa Karinen

CHRONIC CHLAMYDIAL INFECTION: IMPACT ON HUMAN REPRODUCTIVE HEALTH

REPRODUCTIVE HEALTH RESEARCH IN THE NORTHERN FINLAND 1966 BIRTH COHORT (NFBC1966)

FACULTY OF MEDICINE,
DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY,
DEPARTMENT OF PUBLIC HEALTH SCIENCE AND GENERAL PRACTICE,
UNIVERSITY OF OULU,
NATIONAL PUBLIC HEALTH INSTITUTE, OULU