SEROLOGICAL EVIDENCE OF AN ASSOCIATION BETWEEN CHLAMYDIAL INFECTION AND CANCER

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Abstract

Epidemiological and experimental studies indicate a causative role of viruses in malignancies. Recently, a link between bacterial infections and the development of cancer has been suggested. The purpose of this study was to evaluate the association between chlamydial infection and cancer. The association between C. trachomatis infection and cervix cancer was analysed in a prospective study. The presence of IgG antibodies to C. trachomatis and C. pneumoniae was determined from the serum samples of 182 Nordic women with invasive cervical carcinoma and 538 matched cancer-free controls by the microimmunofluorescence (MIF) method. Serum antibodies to C. trachomatis were associated with an increased risk for cervical squamous cell carcinoma (SCC) (OR 2.2, 95% CI 1.3-3.5), but not for cervical adenocarcinoma (OR 0.4, 95% CI 0.1-1.7). C. trachomatis serotype G was highly significantly associated with an increased risk for SCC (adjusted OR 6.6, 95% CI 1.6-27). The presence of serum IgG antibodies to more than one serotype of C. trachomatis, on the other hand, also increased the risk of SCC.

The association between C. pneumoniae infection and lung cancer was analysed separately in men and women. C. pneumoniae-specific antibodies and immune complexes (IC) were analysed from 230 Finnish smoking males with lung cancer and their matched controls using serum samples collected before the lung cancer diagnosis. Suggestive chronic C. pneumoniae infection was associated with an increased risk for lung cancer (OR 1.6; 95% CI 1.0-2.3). The risk was increased especially in men younger than 60 years (OR 2.9; 95% CI 1.5-5.4), but not in the older age group (OR 0.9; 95% CI 0.5-1.6).

Chlamydial antibodies and chlamydia-specific ICs were analysed from serum samples of 29 Finnish women with lung cancer and 87 matched cancer-free controls by MIF. The mean follow-up from serum sampling to cancer diagnosis was 6.7 years. IgG class antibodies to C. pneumoniae were common in pregnant Finnish women (66% among cases, 62% among controls), whereas IC-bound C. pneumoniae IgG antibodies were rare. No additional risk for lung cancer in association with chlamydial antibodies was found among women.

The association between chlamydial infections and lymphomas was evaluated in a cross-sectional study. Seventy-two lymphoma patients from Tampere University Hospital and 72 matched controls were selected, and IgG antibodies and ICs to C. pneumoniae and C. trachomatis were analysed from their serum samples by MIF and enzyme immunoassay (EIA). The serological markers suggesting chronic chlamydial infection were associated with an increased risk for malignant lymphoma. The association was most evident for the presence of C. pneumoniae-specific ICs in non-Hodgkin’s lymphoma (OR = 7.3, 95% CI 2.2-25) and appeared to be limited to men.

Infection with C. trachomatis was found to increase the risk of subsequent development of invasive cervical SCC. Chronic C. pneumoniae infection was also found to be a new independent risk factor for lung cancer in males. Serological markers suggestive of chronic chlamydial infection were associated with lymphomas, proposing that chlamydial infection may have a similar role as H. pylori in the pathogenesis of lymphomas.

Keywords: antibodies, lung cancer, cervical cancer, lymphomas
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Oulu, January 2000  
Tarja Anttila
### Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EB</td>
<td>elementary body</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IC</td>
<td>immune complex</td>
</tr>
<tr>
<td>LGV</td>
<td>lymphogranuloma venereum</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MIF</td>
<td>microimmunofluorescence test</td>
</tr>
<tr>
<td>MOMP</td>
<td>major outer membrane protein</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PID</td>
<td>pelvic inflammatory disease</td>
</tr>
<tr>
<td>RB</td>
<td>reticulate body</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>STD</td>
<td>sexually transmitted disease</td>
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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

Although epidemiological and experimental studies suggest a causative role for viruses in the development of malignancy (zur Hausen 1991), bacterial infections have not traditionally been considered as a major cause of cancer. Recently, however, bacteria have been linked to cancer by two mechanisms: induction of chronic antigen exposure and production of carcinogenic metabolites (Oshima & Bartsch 1994, Rosin et al. 1994). The most specific example of the bacterial inflammatory mechanism of carcinogenesis is *Helicobacter pylori* infection. *H. pylori* has been epidemiologically and also by an animal model linked to adenocarcinoma of the stomach by its propensity to cause long-term inflammation (Parsonnet 1999, Watanabe et al. 1998). It has also been shown that *H. pylori* infection precedes the development of mucosa-associated lymphoid tissue (MALT) lymphoma (Isaacson 1999).

Cervical cancer death rates have dropped dramatically over the past 30 years in most developed countries due to PAP screening programs (Am Cancer Society 1994, Läärä et al. 1987). However, cervical cancer is still the second most common cancer in women world-wide. Past infection with oncogenic human papillomaviruses (HPVs) has been established as a major cause of cervical cancer (Schiffman et al. 1993, IARC 1995). In addition, sexual risk taking behaviour (Rotkin 1973, Buckley et al. 1981) has been demonstrated to be risk factor for cervical neoplasia. Today, *Chlamydia trachomatis* infection is the most common sexually transmitted bacterial disease (STD). Besides *C. trachomatis* infection is a marker of sexual activity, also an association between *C. trachomatis* and cervical cancer has been suggested.

Lung cancer is the most commonly diagnosed malignancy throughout the world and the number one cause of cancer death (Hammar 1994). The lung cancer rates among men began to decline slowly in the early 1980s in Finland, but the rates among women are still increasing (Auvinen et al. 1993). The most important etiologic factor of lung cancer is cigarette smoking, being responsible for 80-85% of the lung cancer deaths (Fielding 1985a,b). Furthermore, chronic bronchitis and other previous lung diseases are known risk factors for lung cancer, both in smokers and non-smokers (Osann 1991). *C. pneumoniae* is a common intracellular bacterium that causes pneumonia and other
respiratory infections world-wide (Kuo et al. 1995). Like all chlamydia organisms, C. pneumoniae has a tendency to cause persistent and chronic infections (Kuo et al. 1995) and possibly induce carcinogenesis in the lung through mediators of inflammation.

Lymphomas are malignant neoplasms characterised by the proliferation of lymphoid cells. Among lymphomas, infectious aetiology has been sought for over a century because of clinical manifestations such as fever, chills, and leukocytosis are associated with these diseases. Helicobacter pylori infection has already been associated with lymphomas (Wotherspoon et al. 1991, Parsonnet et al. 1994). Chlamydia are able to multiply in the cells of reticuloendothelial system (Kaukoranta-Tolvanen et al. 1996) and are also associated with the formation of bronchus-associated lymphoma tissue (Kimura 1994). Furthermore, Chlamydia are mitogenic in vitro (Räsänen et al. 1986) and cause in vivo polyclonal lymphoproliferation (Lehtinen et al. 1986). Thus chlamydia may be associated with lymphomas similarly to H. pylori.

In the present study, the role of chlamydial infections in the development of cervical cancer, lung cancer and malignant lymphomas was evaluated by measuring the elevated chlamydial antibodies and circulating immune complexes (IC) in patients and controls. Because malignancies associated bacterial infections can be cured with antibiotics (Bayerdörffer et al. 1995, Roggero et al. 1995), identification of bacterial causes of malignancy could have important implications for cancer prevention.
2. Review of the literature

2.1. Infections and carcinogenesis

Chronic inflammation associated with many chronic infections may predispose to the development of malignancies. Epidemiological and experimental data indicate a causative role of viruses in malignancies. Viruses can promote the development of human tumours by different mechanisms. Without persistent viral DNA, viruses may induce immunosuppression or modify the host cell genome indirectly. Directly they can induce oncoproteins or alter the expression of host cell proteins at the site of viral DNA integration (zur Hausen 1991). Papillomavirus, hepatitis B virus (HBV), Epstein-Barr virus (EBV) and human T cell leukemia virus (HTLV) infections are associated with human cancers being responsible for about 15 percent of the cancer incidence worldwide. Among cervical and hepatocellular carcinomas, approximately 80 % are linked to viral infections. Thus, viruses must be considered the second most important risk factor for cancer after smoking (zur Hausen 1991).

Papillomaviruses cause benign epithelial proliferation, but have also been associated with both animal and human cancers. Specific types of anogenital HPV infections are at high risk for malignant conversions (HPV 16 and HPV 18) but infections by low-risk types seldom lead to invasive tumours (HPV 6 and HPV 11). Different high-risk serotypes also lead to different types of cervical carcinoma; HPV 16 is common in squamous cell carcinoma, whereas HPV 18 is common in cervical adenocarcinoma (Iwasawa et al. 1996, Dillner et al. 1997).

Epidemiological data also indicate a link between chronic HBV infections and hepatocellular carcinoma (Trichopoulos et al. 1976, Szmuness 1978). Chronic HBV infections are a highly significant risk factor for cancer development, which is especially evident in high-risk areas for hepatocellular carcinoma (zur Hausen 1991, Kew 1998). Vaccination against HBV has been found to decrease hepatocellular carcinoma in these areas (Chang et al. 1997).

EBV has been associated with at least four different types of human malignant tumours: Burkitt’s lymphoma, nasopharyngeal carcinoma, other B-cell lymphomas and Hodgkin’s lymphoma (zur Hausen 1991).
HTLV-I has been linked to an endemically clustered form of leukemic lymphoma, T-cell leukemic lymphoma, which is prevalent on the coast of southern Japan, in the Caribbean and in some parts of central Africa (zur Hausen 1991).

The link between bacterial infections and the development of malignancy is less clear than the link between tumour viruses and malignancy. Among bacterial pathogens, *H. pylori* has recently been associated with gastric carcinoma (Correa *et al.* 1990, Forman *et al.* 1990) and lymphoma (Wotherspoon *et al.* 1991, Parsonnet *et al.* 1994). Besides epidemiological studies, an animal model has confirmed that long-term infection with *H. pylori* induces gastric carcinogenesis (Watanabe *et al.* 1998). Furthermore, some findings also suggest that the effect of specific *H. pylori* strains on tumor development varies by anatomical site (Blaser *et al.* 1995, Chow *et al.* 1998).

Not only viruses and bacteria, but also parasites has been associated with carcinogenesis (Rosin *et al.* 1994). Parasitic disease, schistosomiasis, induce chronic infection in urinary bladder and colorectum and is associated with increased risk cancer at these sites (Rosin & Hofseth 1999). Of the five species of *Schistosoma* that infect humans, *S. haematobium* and *S. japonicum* infections support a causal relationship for development of urinary bladder and colorectal cancer, respectively (Rosin & Hofseth 1999).

Mycoplasma-like organisms have been suggested to be associated with Hodgkin’s disease (Sauter 1995, Johnsson *et al.* 1996). These organisms are intracellular bacteria similar to *Chlamydiae*, and both were originally identified within intraocular leukocytes (Mårdh *et al.* 1989, Wirotsko *et al.* 1993). Recently, an observation that suggests an association between *C. pneumoniae* and cutaneous T-cell lymphoma has been published (Abrams *et al.* 1999).

Bacterial infections may be associated with the development of malignancy by genetic damage and neoplastic transformation, which can be induced *in vitro* by co-culturing cells with activated inflammatory cells (Rosin *et al.* 1994). It has been suggested that nitric oxide and other free radicals released by activated inflammatory cells play a role in carcinogenesis (Oshima & Bartsch 1994). Release of nitric oxide also occurs in *C. trachomatis* infections (Mayer *et al.* 1993). Already in the 1930s, the LGV biovar of *C. trachomatis* causing chronic rectal inflammation, was associated with rectal cancer (Levin *et al.* 1964). Recently, *C. trachomatis* specific DNA has been detected in cervical carcinoma tissues (Schlott *et al.* 1998), and *C. trachomatis* has been shown to inhibit host cell apoptosis by specific mechanisms (Fan *et al.* 1998). Furthermore, *Chlamydiae* are mitogenic *in vitro* (Räsänen *et al.* 1986) and cause *in vivo* polyclonal lymphoproliferation (Lehtinen *et al.* 1986). Chronic chlamydial infections may thus promote malignant transformation.

### 2.2. General aspects of *Chlamydiae*

#### 2.2.1. Taxonomy

*Chlamydiae* are obligate intracellular gram-negative bacteria. They were at first considered as viruses because of their unique biphasic intracellular life cycle (Grayston &
Wang 1975). Thus far, four species of the genus *Chlamydiae* have been identified: *C. trachomatis*, *C. pneumoniae*, *C. psittaci*, and *C. pecorum*. The characteristics of the three main chlamydia species are presented in Table 1. Due to defects in energy metabolism, such as an inability to synthesise many major metabolites, including ATP (McClarty 1999), *Chlamydiae* are dependent on the host cell for their replication. The chlamydial organism is surrounded by an outer membrane which is mainly composed of a major outer membrane protein (MOMP) (Caldwell *et al.* 1981). The outer membrane is very similar to that of many other gram-negative organisms, but differs especially with regard to the amount of peptidoglycan, which *Chlamydiae* synthesise only in small amounts (Stephens *et al.* 1998). Therefore, beta-lactam antibiotics, such as penicillin, have no bactericidal activity against *Chlamydiae*. However, it seems that *Chlamydiae* contain some penicillin-binding proteins, because penicillin can interrupt the normal chlamydial development cycle (McClarty 1999).

*C. trachomatis* species have been divided into 18 serotypes or serovars (Wang & Grayston 1970, Grayston & Wang 1975, Wang *et al.* 1985), according to the *Omp 1* gene that encodes MOMP. At the moment, typing of the *Omp 1* gene is used to classify *C. trachomatis* strains. Further, *C. trachomatis* serotypes can be divided into three biovars: trachoma, lymphogranuloma venereum (LGV) and mouse pneumonitis (MoPn). The trachoma biovar includes the serotypes A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J and K, the LGV biovar the serotypes L1, L2, L2a and L3, but MoPn is a single serovar type (Wang & Grayston 1970, Grayston & Wang 1975, Wang *et al.* 1985). The number of serotypes within the *C. psittaci* species is unknown, but it is genetically more heterogeneous than *C. trachomatis* (Grillner 1991). *C. pecorum*, which causes infections in ruminants, was established as a species distinct from *C. psittaci* (Fukushi & Hirai 1992). Recently, studies on the possible specific relationships with the *Chlamydiae* have highlighted the need for a assessment of the taxonomy of chlamydia-related organisms (Everett *et al.* 1999). This is currently under discussion.

So far, it seems that *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).
Table 1. Essential features of three chlamydial species.

<table>
<thead>
<tr>
<th>Feature</th>
<th>C. pneumoniae</th>
<th>C. trachomatis</th>
<th>C. psittaci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural hosts</td>
<td>Humans</td>
<td>Humans</td>
<td>Birds and lower 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>Epithelial cells, Mononuclear lymphocytes</td>
</tr>
<tr>
<td>Major human disease</td>
<td>Pneumonia</td>
<td>Trachoma</td>
<td>Pneumonia</td>
</tr>
<tr>
<td></td>
<td>Upper respiratory tract infection, Atherosclerosis?</td>
<td>Cervicitis, urethritis, PID, neonatal infection, Reactive arthritis</td>
<td></td>
</tr>
<tr>
<td>Transmission</td>
<td>Aerosol</td>
<td>Sexual, neonatal, Hand to eye, Flies (trachoma)</td>
<td>Aerosol, Excretion</td>
</tr>
<tr>
<td>Number of serovars</td>
<td>1 (? )</td>
<td>18</td>
<td>?</td>
</tr>
<tr>
<td>DNA homology with C. pneumonia (%)</td>
<td>94-100</td>
<td>&lt;5</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

(Source: Schachter 1989, Kuo et al. 1995)

2.2.2. Biology of chlamydia

2.2.2.1. Growth cycle

The biphasic developmental cycle of chlamydia has been described in detail in several studies (Schachter 1989, Kuo et al. 1995). The infective, invasive elementary body (EB) survives extracellularly and the non-infective reticulate body (RB) intracellularly. EBs can enter eukaryotic cells through a phagosome. However, the precise mechanism by which EBs attach to and enter the host cell is unknown (Ward 1995, Peeling & Brunham 1996). EBs reorganise into metabolically active developmental forms, RBs, which are capable of dividing in intracellular vesicle (Schachter 1989, Ward 1995). Towards the end of the life cycle, RBs mature to a new generation of EBs capable of infecting other cells. About 24-72 hours after the attachment of the initial EBs to the host cell, these new infectious EBs are liberated by cell lysis or exocytosis and become able to initiate a new cycle. Besides nutrient deficiency, also antimicrobial agents such as penicillin, may disturb the normal developmental cycle resulting chlamydia to form large abnormal RBs (Beatty et al. 1994a, Ward 1995). These aberrant RBs are unable to divide and develop into EBs and they may persist inside the host cell until the extracellular deleterious factors are removed and the external condition is more favourable.
2.2.2.2. Structural characteristics

The MOMP of a microbe is usually highly antigenic. MOMP is the most prominent component of the chlamydial outer membrane, comprising about 60% of the protein content (Caldwell et al. 1981, Ward 1995). MOMP is a transmembranic protein with type, subspecies, species and genus-specific epitopes defined by monoclonal antibodies (Campbell et al. 1990a, Kuo et al. 1995). The MOMP of C. pneumoniae is more homogenous and less immunogenic than that of the other chlamydial species (Campbell et al. 1990b). Other outer membrane proteins such as the cysteine-rich 60kDa protein, (Omp2) and the small cysteine rich protein, the 12-15 kDa protein (Omp3) (Newhall & Jones 1983, Hatch et al. 1984), can be found in smaller amounts.

The outer membrane also contains lipopolysaccharide (LPS), which is an endotoxin generally found in gram-negative bacteria. Chlamydial LPS is notably similar to the core (Re) of the LPS of Enterobacteriaceae (Nurminen et al. 1983). However, the structure of chlamydial LPS is not identical between the species and the endotoxin activity of chlamydial LPS is much lower than of the LPS of enterobacteria (Nurminen et al. 1983, Brade et al. 1987, Ingalls et al. 1995).

Heat shock proteins (Hsp) are common to all cellular organism. Exposure to a variety of environmental stresses induces Hsps in cells (Zugel & Kaufman 1999). Hsps function as molecular chaperones, aid in antigen presentation and regulate steroid receptor function. Several Hsps have been found in chlamydial cell walls: 75 kDa DnaK-like Hsp70, 60 kDa GroEL-like Hsp60 and the recently defined 17 kDa GroES-like protein Hsp10 (La Verda et al. 1999, Peeling & Mabey 1999). Especially clamydial Hsp60, but also Hsp70 have been implicated important in the immune pathology of chlamydial infections (Peeling & Mabey 1999).

Relatively little is known about the biology of C. trachomatis and even less about C. pneumoniae. However, a comparison of the C. trachomatis and C. pneumoniae genomes will provide some knowledge of common clamydial biological processes required for infection and survival in mammalian cells and difference between the two species in the disease spectrum. Chlamydiae have a rather small genome, approximately 10⁶ base pairs. Recently, the C. pneumoniae genome and two C. trachomatis genotypes have been sequenced (Stephens et al. 1998, Kalman et al. 1999). The C. trachomatis genome consists of a 1 042 519-bp chromosome and a 7 493-bp plasmid (Stephens et al. 1998) whereas the C. pneumoniae genome is larger than that of C. trachomatis consisting of a 1 230 230-bp chromosome (Kalman et al. 1999) with no plasmid present in this strain. The sequences are now publicly available on the internet (http://chlamydia-www.berkeley.edu:4231).

2.2.3. Pathogenetic mechanisms of chlamydial infections

Chlamydiae are responsible for a wide variety of human and animal infections and have a tendency to cause recurrent, persistent or chronic infections (Schachter 1989, Kuo et al. 1995, Ward 1995). However, there are no typical clinical symptoms or features which could differentiate chlamydial from other infections.
Chlamydial species and biovars infect different hosts and different cell types. *C. trachomatis* and *C. pneumoniae* strains are considered strictly human pathogens with no known animal reservoir (Schachter 1989, Kuo *et al.* 1995). However, it seems that *C. pneumoniae* of other mammals will be discovered and *C. pneumoniae* may be more diverse and widespread species than the current literature suggests (Girjes *et al.* 1994). *C. psittaci* infects a wide variety of avian and mammalian species, including humans. On the other hand, *C. pecorum* seems to be exclusively an animal pathogen (Schachter 1989, Fukushi & Hirai 1992). The trachoma biovars A-K of *C. trachomatis* infect primarily columnar but not squamous epithelium, causing mucosal infections, whereas, LGV strains of *C. trachomatis* and *C. pneumoniae* and *C. psittaci* are invasive (Schachter 1989). In *vitro* studies, *C. pneumoniae* has been shown to multiply in macrophages, vascular endothelium and smooth muscle cells, thus making its systemic dissemination through the circulation possible (Kaukoranta-Tolvanen *et al.* 1994, Godzik *et al.* 1995, Gaydos *et al.* 1996).

No long lasting protective immunity for chlamydia develops during acute infections. Thus, *Chlamydiae* may cause repeat infections (Grayston *et al.* 1985, Saikku 1992). Several studies have indicated that chlamydial infections may persist for a long time in humans. However, there is only little evidence about the clinical significance of the persistent chlamydia infections (Beatty *et al.* 1994b, Ward 1995). The persistence of the bacterium has also been established *in vitro* by interrupting the normal development cycle of the bacteria with antibiotics, nutrient-deficient conditions and immune-system regulating factors (Beatty *et al.* 1994b). It has been suggested that the immune response may be ineffective in eradicating chlamydia and protecting the host from reinfections. In humans and monkeys, for example, recurrent infection may cause an intense inflammatory reaction, while primary eye infections resolve with little or no residual tissue damage (Patton & Taylor 1986, Schachter 1989). 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.* 1994a, Ward 1995).

The sequel of unsuspected silent infections associated with the genetically restricted host response are possibly due to Hsps. Repeated or prolonged exposure to Hsp antigens may cause a strong host response against bacterial Hsps and self-Hsps homologous to the bacterial ones (Peeling & Mabey 1999). Therefore, the immune response against these conserved sequences of the Hsps shared by the microbe and the host might potentially lead to an autoimmune reaction (Zugel & Kaufman 1999). Hsp60 seems to be the key antigen in chronic chlamydial infections. It is produced in chronic infections and has been associated with the hypersensitivity phenomenon and the immunopathology seen in these infections (Morrison *et al.* 1989, Peeling & Mabey 1999). Molecular mimicry between bacterial and viral proteins and endogenous molecules, such as the chlamydial Hsp60 antigen and its human homologue (Brunham & Peeling 1994, Paavonen *et al.* 1994), has been implicated to have a role in exacerbation ongoing autoimmune process (Peeling & Mabey 1999).

*C. trachomatis* has been shown to persist in an unculturable intracellular state, in which the synthesis of structural proteins is greatly reduced but the Hsp60 production actually increases (Beatty *et al.* 1993). Several studies have indicated that an enhanced immune reaction against *C. trachomatis* Hsp60 is more typically associated with chronic upper genital tract conditions, including ectopic pregnancy, chronic pelvic pain,
perihepatitis, tubal factor infertility and fallopian tube damage than with acute infections of the lower genital tract (Peeling & Mabey 1999). The scarring tissue damage in trachoma is also connected to Hsp60 targeted immune responses (Peeling & Mabey 1999). Hsp10 reactivity may further contribute to the immunopathologic manifestations of severe upper genital tract complications of chlamydial disease in women (La Verda et al. 1999).

Chlamydic Hsp60 has been associated with the severity of the chronic sequelae of not only *C. trachomatis* infections but also other chlamydial infections. Hsp60 has been localized in human atheromatous tissue (Kol et al. 1998), and associated with the development of atherosclerosis (Xu et al. 1993). Recently, chlamydial infections and heart diseases were shown to be linked by antigenic mimicry (Bachmaier et al. 1999).

Interestingly, an antibody against the chlamydial Hsp70 protein has been shown to neutralise chlamydia infectivity *in vitro* (Danilition et al. 1990) suggesting that the antibody is associated with protective immunity.

### 2.2.4. Clinical spectrum of *C. trachomatis* infection

*C. trachomatis* causes trachoma, which continues to be an important cause of blindness in some parts of the developing world. In these areas, poor economic conditions predominate and young children are frequently exposed to *C. trachomatis*, being the main reservoir of the organism. Primary *C. trachomatis* infection is generally regarded as benign and self limiting, though healing may take months (Beatty et al. 1994b). Persistent and recurrent chlamydial infections leads to scarring of the conjuctiva, and disease severity seem to be associated with repeated *C. trachomatis* infections (Grayston et al. 1985; Bobo et al. 1997), which may ultimately cause blindness many years later (Beatty 1994b, Ward 1995). Certain highly virulent *C. trachomatis* serotypes (Bobo et al. 1997) may also be responsible for the more severe ocular forms of trachoma. Therefore, trachoma is considered a prototype of chronic chlamydial infection. It is the leading cause of preventable blindness in the world. *C. trachomatis* is also the most common cause of neonatal conjuctivitis and one of the most common causes of pneumonia in early infancy (Black 1997).

Although *C. trachomatis* still is 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. The World Health Organisation estimates that about 90 million of all new STD infections are caused by *C. trachomatis*. The highest rates are found in young, sexually active populations (WHO 1996). Compared to older females, young women often have cervical ectopy, where the squamocolumnar junction, a primary host target for *C. trachomatis*, is everted and thus more exposed to Chlamydiae.

Most infections caused by *C. trachomatis* in women are asymptomatic. However, clinical manifestations include cervicitis, urethritis, endometritis, pelvic inflammatory disease (PID), and abscess of the Bartholin’s glands (Stamm et al. 1999). The initial site of infection is usually the cervix, but the urethra and the rectum may also be infected (Stamm et al. 1980). Culture studies have shown that approximately half of the women
with *C. trachomatis* are infected at both the cervix and the urethra, while one third have only cervical infections, and 5 to 30% have only urethral infections (Paavonen 1979, Paavonen *et al.* 1982, Phillips *et al.* 1987). Lower genital tract infections, urethritis and cervicitis, are completely asymptomatic or carry a wide range of symptoms (Paavonen 1979, Stamn *et al.* 1980, Cates & Wasserheit 1991, Horner *et al.* 1995). Most women with chlamydial cervicitis have minimal symptoms (Grayston & Wang 1975, Paavonen 1979, Cates & Wasserheit 1991).

The predominant *C. trachomatis* serotypes in urogenital tract infections are the serotypes D, E, and F (Wang *et al.* 1985, Saikku & Wang 1987, van Duynhoven *et al.* 1998). In women, serotype G has been associated with symptomatic infection (Lan *et al.* 1995) and the serotypes D and F with asymptomatic infection (Workowski *et al.* 1994, Lan *et al.* 1995). Serotype E has been found both symptomatic and asymptomatic women (Dean *et al.* 1995, Lan *et al.* 1995). Furthermore, Dean *et al.* (1998) have shown that almost all patients with repeat *C. trachomatis* infection are infected with uncommon C complex serotypes, suggesting that the C complex is associated with chronic or recurrent infections.

*C. trachomatis* infection may persist subclinically in the endometrium for a long time (Paavonen *et al.* 1985a,b) and produce chronic subclinical infection analogous to trachoma. The presence of plasma cells in the endometrial stroma [i.e. plasma cell endometritis (PCE)] is characteristic to chronic endometritis (Greenwood & Moran 1981, Kiviäit *et al.* 1990). *C. trachomatis* has been reported as a causative agent of PCE cases (Kiviäit *et al.* 1986, Paavonen *et al.* 1987, Paukku *et al.* 1999) and also associated more often in severe PCE with lymphoid follicles (Paavonen *et al.* 1985b) than non-chlamydial endometritis.

Women with chlamydia isolated from the cervix often show no signs or symptoms of infection. On examination, however, at least a third generally have local signs of infection, such as endocervical bleeding, mucopurulent endocervical discharge, and edema within the area of ectopy (Paavonen *et al.* 1988). It has been reported that colposcopic features of immature squamous cell metaplasia of the cervix is associated with chlamydial infection (Paavonen *et al.* 1988). The number of polymorphonuclear leukocytes in cervical mucus also correlates with chlamydial infection of the cervix (Kiviäit *et al.* 1985). Finally, patients with cervicitis caused by *C. trachomatis* are at risk for further development of PID (Paavonen *et al.* 1985a, Paavonen *et al.* 1987, Hillier *et al.* 1996).

PID has been defined as a syndrome associated with spreading of micro-organisms from the vagina and cervix to the endometrium, salpingeal tubes and adjacent structures (Weström 1980). Today, the majority of PID episodes of known aetiology are caused by *C. trachomatis* (Paavonen *et al.* 1987, Heinonen & Miettinen 1994, Paavonen & Lehtinen 1996, Paavonen 1998). A large proportion of *C. trachomatis* infections in the salpingeal tubes are asymptomatic, subclinical or atypical, and difficult to recognise as PID. With repeated infections, the risk of PID increases (Hillis *et al.* 1997).

In tubal infection, the fibrosis and scarring (Weström 1994, Paavonen & Lehtinen 1996) lead to permanent tubal damage, which increases the risk of ectopic pregnancy (Hillis *et al.* 1997) and tubal factor infertility (Weström 1980). The more episodes of PID one has had, the higher is the risk for infertility (Weström 1980, Paavonen & Lehtinen 1996).
Pregnant women with chlamydial infections are at an increased risk for adverse outcomes of pregnancy and postpartum endometritis (Smith & Taylor-Robinson 1993, Claman et al. 1995, Paavonen & Lehtinen 1996). *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. *C. psittaci* infection is also suggested to cause abortion by inducing acute inflammatory response in the placenta (Roberts et al. 1967, Johnson et al. 1985, Flanagan et al. 1996).

LGV is a sexually transmitted systemic infection caused by *C. trachomatis* strains L1, L2 and L3. It is uncommon in industrialised countries but frequent in parts of Africa, Asia and South America. It predominantly infects lymphatic tissue (Schachter & Osoba 1983), 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). Acute LGV is reported over five times more frequent in men than in women (Schachter 1977). LGV has various acute and late manifestations. Most of the patients recover from LGV without late sequel. In some patients, however, the persistence of chlamydia in anogenital tissue may induce a chronic inflammatory response and may cause an anogenital syndrome with subacute manifestations, such as proctocolitis and hyperplasia of intestinal and perirectal lymphatic tissue. Perirectal abscesses, ichtiocrectal and rectovaginal fistulas, anal fistulas and rectal stricture or stenosis are chronic or late manifestations of LGV. Antibiotic treatment during the second stage of LGV, i.e. inguinal or anogenital syndrome, prevents the late complications of the disease (Perine & Stamm 1999).

### 2.2.5. Clinical spectrum of *C. pneumoniae* infection

*C. pneumoniae* infections occur world-wide both endemically and epidemically, and the prevalence varies from one region to another. *C. pneumoniae* is primarily a human respiratory pathogen (Saikku et al. 1985, Grayston et al. 1986, Ekman et al. 1993a, Kuo et al. 1995), and it is probably transmitted from person to person by respiratory secretions (Grayston et al. 1986, Grayston et al. 1990, Mordhorst et al. 1994). Transmission usually takes place outside home. Closed communities, such as military garrisons, schools and large families, have an important role in pneumonia outbreaks. The incubation time is around 3 weeks (Mordhorst et al. 1994). The infection spreads inefficiently, and perhaps only a few infected persons transmit the organisms. However, transmission may also occur through asymptomatic carriers (Kleemola et al. 1988).

*C. pneumoniae* infections occur yearly, however, cyclic variations has been shown in the incidence: two to three year periods of high incidence is followed by 3- to 10- year periods of low incidence (Grayston et al. 1990, Karvonen et al. 1993). In children in tropical countries, *C. pneumoniae* infection is more common and more severe (Saikku et al. 1988a) than in the developed countries, where very few patients under five years of age have serological evidence of past infection. The prevalence increases clearly after the age of 5 years, and approximately half of the population aged 20 years have antibodies against the organism. 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, while in the case of *C. trachomatis*, antibody prevalence clearly falls after 40 to 50 years of age. This indicates that most people have two or three *C. pneumoniae* infections during their lifetime or alternatively a possible persistent *C. pneumoniae* infection (Grayston *et al.* 1990, Saikku 1992, Kuo *et al.* 1995). The *C. pneumoniae* antibody prevalence is also higher in smokers than among non-smokers (Karvonen *et al.* 1994).

The clinical features of *C. pneumoniae* infections are not typical. Apart from pneumonia, the most frequent illness associated with *C. pneumoniae* is bronchitis (Grayston & Wang 1975, Kuo *et al.* 1995). Most infections are subclinical (Kleenmola *et al.* 1988), and clinical symptoms and pneumonia are more frequent in patients older than 20 years of age (Ekman *et al.* 1993a). Among teenagers and young adults, pneumonia or prolonged bronchitis is caused by a primary infection, and patients are often febrile and hoarse (Grayston *et al.* 1990, Kuo *et al.* 1995).

Respiratory illnesses caused by *C. pneumoniae* seldom require hospitalisation, as the infection is mostly relatively mild and patients usually respond to antimicrobial treatment. However, in patients with COPD (Kauppinen & Saikku 1995) and particularly in older people (Kauppinen *et al.* 1995, Peeling *et al.* 1997), *C. pneumoniae* pneumonia may be severe and complete recovery may be slow regardless of antibiotic therapy (Kuo *et al.* 1995, Peeling *et al.* 1997). Pharyngitis, laryngitis, sinusitis and otitis media are also caused by *C. pneumoniae* (Kuo *et al.* 1995).

Several chronic inflammatory diseases involving both respiratory and non-respiratory organs have been associated with *C. pneumoniae* infections. Chronic inflammatory conditions of the respiratory tract are logical consequences of *C. pneumoniae* infections. High prevalence of IgG antibodies and local sputum IgA antibodies to *C. pneumoniae* have been observed in patients with chronic bronchitis, suggesting a chronic respiratory *C. pneumoniae* infection (von Hertzen *et al.* 1996). The onset of asthma and asthma exacerbations have also been proposed to occur in association with *C. pneumoniae* infection (Hahn & Allegra 1999). Smoking has been identified as the main risk factor for the development of chronic bronchitis and COPD; most of the patients are elderly male smokers. Serological studies have shown that *C. pneumoniae* is involved in 4-5% of acute exacerbations of COPD (Blasi *et al.* 1993). Stable elevated IgA antibodies in sputum and frequent presence of circulating ICs, the markers of chronic *C. pneumoniae* infection, may reflect a defence mechanism mitigating airway inflammation (von Hertzen *et al.* 1997).

Even before the association of *C. pneumoniae* infection with chronic respiratory diseases was recognised, a connection between *C. pneumoniae* infection and atherosclerosis was discovered (Saikku *et al.* 1988b). Saikku *et al.* showed (1988) that patients with acute myocardial infarction (AMI) and coronary heart disease (CHD) had more often elevated *C. pneumoniae* IgG and IgA antibody levels than healthy controls. Later, the same investigators showed that AMI and CHD patients had circulating ICs containing chlamydial LPS or *C. pneumoniae* protein-specific ICs present in their sera (Leinonen *et al.* 1990, Saikku 1992, Linnanmäki *et al.* 1993). So far, the serological association between *C. pneumoniae* and atherosclerosis has been confirmed in approximately thirty studies (reviewed by Saikku 1997, Campbell *et al.* 1998). In addition, the presence of *C. pneumoniae* antigens and nucleic acid has been demonstrated in atherosclerotic lesions (Kuo *et al.* 1993, Kuo *et al.* 1995).
Sarcoidosis, hilar lymphadenopathy and reactive arthritis (Kuo et al. 1995) have also been associated with *C. pneumoniae* infection.

### 2.2.6. Diagnosis of chlamydial infection

#### 2.2.6.1. Culture

Culture has been the golden standard in chlamydial diagnosis. Since chlamydia is an intracellular organism and requires careful specimen transportation, a high level technical expertise and time-consuming incubation (3 to 7 days), the method involves many difficulties. Culture has a specificity that approaches 100%, but it is relatively insensitive being only 50%-85% compared to DNA amplification tests (Black 1997, Peeling 1999).

#### 2.2.6.2. Antigen detection

The present antigen detection methods are based on the demonstration of genus-specific chlamydial LPS and cannot differentiate between chlamydial species (Black 1997, Peeling 1999). For antigen detection, the presence of viable *Chlamydiae* is not required and it may therefore be useful in the diagnosis of chronic chlamydial infections (Saikku 1994), if sufficient amounts of antigens are present (Beatty *et al.* 1994b).

The direct fluorescence antibody technique (DFA) adds the advantage of chlamydia-specific antibody staining to the direct examination of clinical specimens. Although the DFA staining method is rapid, the microscopic evaluation of each specimen is laborious and requires highly trained and experienced personnel (Black 1997, Peeling 1999).

Enzyme immunoassay (EIA) designed for *C. trachomatis* can also be used for the detection of the *C. pneumoniae* antigen, since the capture antibody in chlamydia 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).

#### 2.2.6.3. Nucleic acid amplification

The development of the nucleic acid amplification tests has been the most important advance in the field of chlamydial diagnosis and they will replace the culture of the organism from clinical specimens. Nucleic acid amplification tests has been used to detect *C. trachomatis* in first-void urine specimens and vaginal swabs (Schachter *et al.* 1995, Stary *et al.* 1997) and *C. pneumoniae* on sputum (von Hertzen *et al.* 1997), in circulating, purified white blood cells (Boman *et al.* 1998) and in tissues (Kuo *et al.* 1995).
The most widely known DNA amplification technology is polymerase chain reaction (PCR). Two synthetic oligonucleotide primers are used in PCR test. The primers have sequences that are complementary to flanking regions of a specific DNA segment of the target organism. Depending on primer design, chlamydia PCR can be genus, species, group, or strain-specific. The lower detection limits in most of these methods are 5 to 100 EBs (Black 1999, Peeling 1999). The specificity of the method is 95-100% for both C. pneumoniae and C. trachomatis (Black 1997, Peeling 1999). Due to inhibitory factors, the sensitivity of PCR has been variable. However, the method is estimated to be more sensitive than culture, mainly because no stringent specimen transport conditions are required for PCR (Kuo et al. 1995, Black 1997, Peeling 1999).

Several C. pneumoniae PCR procedures are based on target sequences in 16S ribosomal DNA, the major outer membrane and others (Campbell et al. 1992, Gaydos et al. 1993, Tong & Sillis 1993). Also a commercial automated PCR assay for C. trachomatis (Cobas Amplicor C. trachomatis test, Roche Diagnostic Systems Inc., Branchburg, N.J.; Loeffelholz et al. 1992) is already available for routine use. In the Amplicor test, primers target a 207-bp segment of the cryptic plasmid DNA present at 7 to 10 copies per genome of C. trachomatis strains. This makes plasmid PCR more sensitive than PCR based on the detection of the MOMP gene, which involves only 1 copy per genome (Black 1997). Clinical specimens are known to contain several factors that inhibit DNA polymerase. However, a system that permits the identification of such factors has been developed, and the detection of the amplification of an internal control can be included in the C. trachomatis PCR test (Roche Diagnostic) to ensure the integrity of the result.

Ligase chain reaction and transcription mediated amplification are other nucleic acid amplification tests used for the diagnosis of C. trachomatis infection (Black 1997).

2.2.6.4. Serology

Complement fixation (CF). In the CF test, the target of the antibodies is genus specific LPS; thus it is not possible to determine the species-specific antibody response with this test. Although the CF test lacks specificity, it is technically much easier than the microimmunofluorescence (MIF) test and has objective end-points. The treatment with antibiotics may delay or diminish the production of CF antibodies (Black 1997) decreasing the sensitivity of the test.

Enzyme immunoassay (EIA). EIA kits with LPS-extracted EBs of C. pneumoniae or C. trachomatis as antigen are commercially available for the detection of chlamydial antibodies. EIA tests are generally sensitive, but problems with sensitivity and specificity have been encountered with these kits (Peeling 1999, Black 1997). Recently, EIA tests that apply C. trachomatis MOMP variable domain IV synthetic peptides as antigen, have been developed (Närvänänen et al. 1997). Because EIA tests based on synthetic peptides are also antigen-site specific (Norby et al. 1987) they can therefore discriminate antibody responses even against different chlamydial immunotypes (Jones et al. 1992).

Microimmunofluorescence test (MIF). The MIF test was developed in the early 1970s as a tool for epidemiologic research on chlamydial infections (Wang & Grayston 1970).
The MIF test is able to differentiate between both chlamydial species and serotypes as well as subclasses of antibodies (Wang & Grayston 1970, Kuo et al. 1995, Anttila et al. 1998). If performed and read properly, this test provides a sensitive and most specific method for the laboratory diagnosis of chlamydial infection. In the case of acute chlamydial infection, the criterion for a serological diagnosis is a four-fold rise in the IgG titre or IgA or single IgM titre ≥ 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 chlamydia 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 the MIF test in primary infections (Saikku 1994). In reinfections, IgG and IgA titres rise quickly, i.e. in 1-2 weeks without an IgM response (Grayston et al. 1990).

Both elevated short-lived IgA antibodies and microbe-specific ICs have been shown to persist in chronic C. pneumoniae infections (Saikku 1992, Saikku 1999; Table 2). The presence of persistent ICs in serum may reflect continuous production of microbial antigens (Saikku 1994). Therefore, in certain circumstances these antibodies may be more reliable markers of chronic chlamydial infection than the presence of IgG (Saikku 1992).

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

<table>
<thead>
<tr>
<th>Chlamydia infection</th>
<th>MIF assay</th>
<th>Immune complexes (IC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute infection</td>
<td>IgM titre ≥ 16</td>
<td>Present in pneumonia</td>
</tr>
<tr>
<td></td>
<td>Four-fold rise in IgG titre</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Four-fold rise in IgA titre</td>
<td></td>
</tr>
<tr>
<td>Chronic infection</td>
<td>Persistent presence of elevated IgG and IgA antibodies</td>
<td>Persistent presence</td>
</tr>
</tbody>
</table>

2.3. Cervical cancer

Although the mortality from cervical cancer has dropped clearly over the past 30 years (Am Cancer Society 1994, Läärä et al. 1987) it still is the second most common cancer in women world-wide (Parkin et al. 1988). The median age at which carcinoma of the cervix is diagnosed is 48 years i.e, about 20 years younger than the median age of the diagnosis of carcinoma of the vagina, vulva or anus. The incidence of invasive cervical cancer increases with age, rising sharply to 15/100 000 among premenopausal women (20 to 35 years), and after that being around 15-20/100 000 (Kiviati et al. 1999).

Past infection with oncogenic HPV, particularly the HPV types 16 and 18, has been established as a major cause of cervical cancer (Schiffman et al. 1993, IARC 1995). Squamous cell carcinoma is primarily associated with HPV16 and adenocarcinoma with HPV18 (Dillner et al. 1997). However, results show that cervical carcinoma patients with no identifiable HPV nucleic acids have a poorer prognosis than those with evidence of
HPV infection (Higgins et al. 1991). Sexual risk-taking behaviour, such as early sexual experiences and multiple partners (Rotkin 1973, Buckley et al. 1981), C. trachomatis infection (Paavonen et al. 1979, Schachter et al. 1982, Hakama et al. 1993, Bosch et al. 1996), birth control pill use (Beral et al. 1988), dietary factors (La Vecchia et al. 1984, Lehtinen et al. 1999) and smoking (Winkelstain 1990) have also been demonstrated or suggested to be risk factors for cervical neoplasia. However, the fact that virgins are well protected against cervical cancer (Ponten et al. 1995) indicates that a sexually transmitted risk factor or risk factors are probably involved in the aetiology of cervical carcinoma.

Invasive cervical cancer is usually preceded by premalignant or dysplastic changes in the cervical epithelium. These lesions are classified as low-grade lesions or high grade lesions. Epithelium adjacent to invasive carcinomas was quite early found to be replaced by a full-thickness layer of cells morphologically identical to invasive tumour cells (Kiviat et al. 1999), called "carcinoma in situ" (CIS). Nowadays, mild, moderate and severe dysplasia and CIS are generally considered to represent a morphologic and biologic continuum of progressive consecutive stages in the development of invasive cancer (Lange 1960, Richart & Barron 1969, Spriggs & Boddington 1980). On the basis of retrospective studies of women with invasive squamous cell carcinoma, in whom prior biopsies showed CIS, it has been universally accepted that such lesions may progress into invasive carcinoma (Kiviat et al. 1999).

Squamous cell carcinoma is the most common type of cervical cancer, accounting for 80-90% of all invasive tumours while adenocarcinoma makes up most of the remaining cancers. Ninety-five percent of cervical cancers originate from the squamocolumnar junction (Bangle et al. 1963, Boyd & Doll 1964), where the squamous epithelium of the ectocervix interfaces to the glandular epithelium of the endocervix. In young girls, glandular epithelium covers large parts of the ectocervix, which condition is called "ectopy", but as adolescence progresses, the junction migrates up towards the endocervical canal. This process is generally regarded to occur through metaplasia, where glandular cells are converted to or overgrown by squamous epithelium. Such actively changing tissue may be more susceptible to multiple insults of cancer causing factors and thus young women are more vulnerable to the effect of antigen exposure. In this respect, increasing attention has recently been given to the view that cervical cancer is a STD (Chamberlain 1981).

Since C. trachomatis infection also is a marker of sexual activity, an association between C. trachomatis and cervical cancer is plausible. As early as about 50 years ago, LGV associated chronic rectal inflammation was linked to rectal cancer (Levin et al. 1964), and today the original suggestion of an association of C. trachomatis infection with cervical neoplasia (Paavonen et al. 1979, Schachter et al. 1982) has been repeatedly confirmed in cross-sectional and longitudinal case-control studies (Hakama et al. 1993, Bosch et al. 1996).

Endocervical glandular cells are known to be the target cells for C. trachomatis. The prevalence of C. trachomatis infection is higher in females with ectopy than in women without it, showing that women with cervical ectopy are more susceptible to C. trachomatis infection. Immature squamous metaplasia on the ectopy zone is associated with chlamydial infection (Paavonen et al. 1988). Cervical ectopy is normally present in 60-80% of sexually active adolescents and then declines in prevalence in the third and fourth decades. This probably explains the high prevalence of cervical chlamydial
infections in adolescents. With increasing age, the endocervical epithelium undergoes metaplasia and metaplastic cells also are target cells for *C. trachomatis*. In fact, persistent chlamydial infection may induce metaplasia (Kiviat *et al*. 1985, Paavonen *et al*. 1988). However, it is not clear whether the observed associations between multiple STDs and cervical cancer are due to a misdiagnosis of HPV exposure or whether non-HPV microorganisms really are significant etiologic cofactors (Schiffman *et al*. 1993).

### 2.4. Lung cancer

Lung cancer is one of the most frequently diagnosed malignancies throughout the world (Hammar 1994). In Finland, about 1,700 new cases of lung cancer (42/100,000) are diagnosed annually among males whereas in the European Community and in the USA the rate is 64/100,000 and 71-80/100,000, respectively. The corresponding figures for females are 8.4, 8.2 and 25.8-42.9, (Finnish Cancer Registry 1997, Parkin & Muir 1992, Zheng *et al*. 1994). In the early 1980s, in Finland the lung cancer rates among men began to decline slowly, while the rates among women are still increasing (Auvinen *et al*. 1993). Lung cancer is the number one cause of cancer death, accounting for 4% of all deaths and 21% of all cancer deaths in Finland (Central Statistical Office of Finland 1985) and for 6% and 28%, respectively, in the USA (Beckett 1993).

Several agents are involved in the aetiology of lung cancer. The most important etiologic factor of lung cancer is cigarette smoking, which is involved in 80-85% of lung cancer deaths (Fielding 1985a,b). Smoking increases the risk of all histologic lung cancer types, but the high dose-dependent risk is stronger for small cell and squamous cell carcinoma than for adenocarcinoma (Morabia & Wynder 1992). This may be explained by the abundant exposure of the proximal parts of the respiratory tract to smoke particles (Morabia & Wynder 1992).

Of the occupational factors, the role of asbestos is important in the development of lung cancer. Epidemiologic (Selikoff *et al*. 1979) and experimental (Barrett *et al*. 1989) studies indicate that asbestos is a carcinogen, and it has been shown to act both as an initiator and a promotor of the development of lung cancer (Barrett *et al*. 1989).

Viral oncogenesis as a possible cause for the increased incidence of peripheral lung cancers has been proposed by Auerbach & Garfinkel (1991). HPV may be a possible etiologic factor in squamous cell and large-cell lung carcinoma (Syrjänen & Syrjänen 1987, Bejui-Thivolet *et al*. 1990, YousOf et al. 1992). The risk factors for lung cancer further include gender, race and genetic predisposition (Sellers 1990, Beckett 1993).

Lung cancers are commonly divided into two main groups based on their histology and clinical features, namely small cell lung cancer and non-small cell lung cancer. The major histologic types of common malignant epithelial neoplasms have been classified as follows: 1) squamous cell carcinoma 2) small cell carcinoma 3) adenocarcinoma 4) large-cell carcinoma and 5) adenosquamous carcinoma (WHO 1981).

Squamous cell carcinoma originates from squamous or metaplastic squamous cells in the bronchial mucosa. It develops first with increasing degrees of atypia and dysplasia, then as an in situ carcinoma and finally as an invasive carcinoma (Saccomanno et al. 1974, Auerbach et al. 1979). Squamous metaplasia probably promotes the proliferation and differentiation of reserve or "basal" cells, but it may also result from direct conversion of columnar mucous cells into squamous epithelial cells (Trump et al. 1978).

Small cell carcinoma of the lung originates from the neuroendocrine cells of the bronchopulmonary tree (Hammar 1994). These tumours are strongly associated with cigarette smoking. Pulmonary neuroendocrine cells multiply in cigarette smoke-associated pulmonary diseases (Hammar 1994). Mutations of the \textit{p53} gene are found in over 75% of small cell carcinomas (D’Amico et al. 1992).

Adenocarcinoma is usually located peripherally and is a heterogeneous type of lung cancer. Whether or not a histological precursor lesion for adenocarcinoma exists is not known (Li et al. 1994). Most of the adenocarcinomas occur in cigarette smokers, and a majority of them are associated with scarring (Hammar 1994). \textit{p53} mutations and protein accumulation have also been found in atypical alveolar epithelial hyperplasia (Kawai et al. 1994).

2.5. Lymphomas

Lymphomas are malignant neoplasms characterised by proliferation of lymphoid cells, i.e. lymphocytes, histiocytes and their precursors and derivatives. Malignant lymphomas comprise a incoherent group of tumours of the immune system. The clinical behaviour of these diseases varies from highly malignant, aggressive to indolent, well-tolerated tumours (Rosenberg 1979). Within the broad spectrum of malignant lymphomas, Hodgkin’s disease (Hodgkin’s lymphoma) is segregated from the other forms, which are classified as non-Hodgkin’s lymphomas. Although both have their origin in lymphoid tissue, Hodgkin’s disease is differentiated by the characteristic Reed-Stenberg giant cells.

The development of most malignant lymphomas is thought to result from errors in lymphocyte transformation, which is essential for the normal differentiation and function of lymphocytes.

2.5.1. Hodgkin’s disease

Hodgkin’s disease constitutes approximately 40% of the malignant lymphomas and is one the most common form of malignancy in young adults. About 50% of the cases occur between the ages of 20 and 40. The incidence of Hodgkin's disease is higher in males (4:2.6), and males also have a poorer prognosis than females (Grufferman 1982). The
aetiology of Hodgkin’s disease is unknown. For years, the EBV has been suspected to be an etiologic agent on the basis of epidemiological and serological studies. Evidence of EBV infection is present in some but not all cases. It has been suggested that Hodgkin’s disease is heterogeneous with respect to both the cell type involved and the aetiology. Therefore, EBV-negative Hodgkin’s disease may be linked to an as yet unidentified (microbial) agent. Since Hodgkin’s disease is associated with certain HL-A antigens, and family members also have an increased incidence, genetic and environmental factors may be involved in the pathogenesis of Hodgkin’s disease (Grufferman 1982).

2.5.2. Non-Hodgkin’s lymphoma

Age at peak incidence is higher in non-Hodgkin’s lymphoma than that in Hodgkin’s disease. About 25% of cases develop in the age range of 50-59 years. Maximal risk occurs between the ages of 60 and 69 years. Incidence is slightly higher in males (1.4:1). The strongest association between malignant lymphomas and environmental factors are that between Burkitt lymphoma and EBV and between adult T-cell lymphomas and HTLV.

A viral aetiology has been suggested by the epidemiological, electron microscopycal, cell culture, and immunological studies, but no causal relationship has been proved (Aisenberg et al. 1973, Klein et al. 1976). Anyway, non-Hodgkin’s lymphoma may be amenable to be controlled by vaccination and immunological means (Miller et al. 1982). The association of lymphocytic and histiocytic malignancies with chronic immuno-suppressive therapy seems compatible with either a viral aetiology or an induced immunologic defect that permits a malignant clone to proliferate (Penn 1975). The theories of lymphomagenesis imply a loss of immune suppressor cell function or an activation of oncogenic viruses in disturbed immune stimulation (Braylan et al. 1975, Lutzner et al. 1975, Berard et al. 1976, Klein et al. 1976, Greene 1982).

2.5.3. MALT-lymphoma

The most common form of extranodal non-Hodgkin’s lymphoma is the gastric lymphoma. The term “mucosa associated lymphoid tissue” (MALT) identifies the presence of lymphocytes in the mucosa of various tissues or organs. The lymphoma typically affects middle-aged subjects and shows an indolent course compared with other lymphomas. Selective proliferation of a clone of B-lymphocytes, B-cell clonality, is the basic diagnostic criterion for MALT lymphoma. It usually results from persistent antigenic stimulation, often due to an infectious or other exogenous agent. Little is known about the pathogenesis, clinical significance and prognosis of gastric MALT lymphoma. It is thought to represent a low-grade lesion that may evolve into a high-grade lymphoma (Isaacson 1994). Some evidence show that MALT lymphoma arises as a result of chronic *H. pylori* infection, and this organism can be found in the gastric mucosa in nearly all cases (Wotherspoon et al. 1991). Eradication of *H. pylori* in patients with low-grade gastric primary B-cell lymphoma may result in tumour regression (Wotherspoon et al. 1993), suggesting that the tumours are directly or indirectly dependent on bacterial stimulation.
3. Objectives of the study

The general purpose of this work was to study the possible association between chlamydial infection and cancer by using serological methods.

The specific aims were:

1. to investigate the serological association between \textit{C. trachomatis} infection and cervical cancer,
2. to investigate the serological association between \textit{C. pneumoniae} infection and lung cancer,
3. to determine the prevalence of serological markers of chlamydial infection in malignant lymphomas.
4. Study populations

4.1. Nordic Serum Banks (I, II)

Serum banks of Finland, Norway and Sweden were used to study the association between \textit{C. trachomatis} infection and cervical cancer. These three population based serum banks contain blood samples from approximately 530,000 women from Finland, Norway and Sweden (described more detailed in study I and II). The chief applicant has a special permission of the Data Inspection Board for linkage of the National Public Health Institute’s Maternity cohort and Finnish Cancer registry data files. For other registry linkages and research on humans special permissions have been applied for from the ethical committee of the Department of Obstetrics and Gynaecology, University of Helsinki Central Hospital. In Norway and Sweden the women and men are invited to participate in a health-promoting project, including donation of biological samples following an informed consent for future medical research. Cases of cervical carcinoma were identified by linking the data files of the Nordic serum banks with the nation-wide cancer registries.

By the end of 1993, 182 women developed invasive cervical carcinoma; 49 were from Finland, 128 from Norway and 5 from Sweden. Of all cases, 149 (82\%) had squamous cell carcinomas, 32 (18\%) adenocarcinomas (including adenosquamous carcinomas), and one unspecified carcinoma. Of the carcinomas, 107 (59\%) were localised, 62 (34\%) were metastatic, and in 13 cases the stage was unknown. The mean age at diagnosis was 44 years (range 23-64), and the mean lag between serum sampling and diagnosis was 56 months (range 1-195).

The earliest available prediagnostic serum sample was chosen. For each case, three cancer-free controls were selected, matched for sex, age at serum sampling (±2 years), storage time of the serum sample (±2 months), country and, in Norway, the county of residence (described more detailed in study I and II).
4.2. ATBC Study (III)

The ATBC (alpha-tocopherol beta-carotene) study, conducted in south-western Finland in 1985-1988 was used to analyse the association between *C. pneumoniae* infection and lung cancer in males in study III (described more detailed in study III). The mean lag between serum sampling and cancer diagnosis was 4 years 11 months. This study was approved by the institutional review boards of the participating institutions (National Public Health Institute, Helsinki, Finland and National Cancer Institute, Bethesda, USA as principal institutions). All subjects provided informed consent before randomisation.

The 230 lung cancer cases and the matched controls were similar in their distribution of age (mean 60.3 years in both), years of regular smoking (mean 40.7 vs. 38.1 years) and daily cigarette consumption (mean 21.5 vs. 19.7). Of the cancers, 99 (46%) were squamous cell carcinomas, 55 (25%) small cell carcinomas, 34 (16%) adenocarcinomas and 28 (13%) other carcinomas; 14 cases had no histological or cytological diagnosis (described more detailed in study III).

4.3. Finnish Maternity Cohort (IV)

The Finnish Maternity Cohort (FMC) was also used in study IV to analyse the association between *C. pneumoniae* infection and lung cancer in females.

Overall 29 cases with histologically confirmed lung cancer were diagnosed by December 31, 1996, as identified through a record linkage of the serum bank data file and the Finnish Cancer Registry. There were 13 adenocarcinomas, 1 adenoid cystic carcinoma, 5 squamous cell carcinomas, 5 small cell carcinomas, and 5 unspecified carcinomas. The mean age of the patients was 32 years at enrolment (range 20-42) and 39 years at the time of diagnosis (range 23-51). The mean lag (elapsed time) between the serum sampling and the diagnosis of cancer was 6.7 years (range 1.6-11.6). Eighty-seven controls individually matched for sex, age (±2 years), and sample storage time (±2 months) were identified from the FMC; sufficient serum samples were available for 84 controls.

4.4. Lymphoma Study (V)

For Study V, serum samples of 72 patients with lymphoma (31 females and 41 males) were obtained from the University Hospital of Tampere in 1989-1992, to investigate the association of chlamydial infections and malignant lymphomas. The study also involved serum samples of 72 healthy (blood donors) controls matched for age (±1 year), sex, residence (county) and time of sample storage (±1 year). Of the patients, 53 had non-Hodgkin's lymphomas and 19 had Hodgkin’s disease. The mean age at diagnosis was 53 years (range 19-74): 57 years in the cases with non-Hodgkin's lymphoma (range 27-74) and 43 years in the cases with Hodgkin’s disease (range 19-69). The serum samples were collected at the time of diagnosis and stored at -25°C until analysed. Permission for the study was applied from the ethical committee of the Tampere University Hospital.
5. Methods

5.1. Chlamydia serology

5.1.1. Microimmunofluorescence test (I-V)

*C. pneumoniae*-specific IgG (I-V) and IgA (III, IV, V) and *C. trachomatis* IgG antibodies were measured by the MIF method. EBs of *C. pneumoniae*, Kajaani 6 strain (I-V), and pooled serovars BED (B-group), CJHI (C-group), and GFK (intermediate group) of *C. trachomatis*, (I, II, IV, V; Washington Research Foundation, Seattle, WA, USA) or strain L2 (III) of *C. trachomatis* were used as antigens. Fluorescein isothiocyanate (FITC)-conjugated antihuman IgG (Kallestad, Chaska, MO, USA) and IgA (Sigma, St. Louis, MO) were used as conjugates. The serum samples were analysed at twofold dilutions for *C. trachomatis* and at fourfold dilutions for *C. pneumoniae*. Titres of $\geq 8$ (V), and $\geq 16$ (I, II, IV) were considered positive for *C. trachomatis* IgG antibodies and titres of $\geq 32$ for IgG and $\geq 16$ for IgA were considered positive for *C. pneumoniae*.

All *C. trachomatis* positive sera detected in study I were further analysed in study II for antibodies against the serotypes B, D, E, G, F, J (Washington Research Foundation, Seattle, WA, USA) and C, H, I, and K (American Type Culture Collection, Rockville, Maryland, USA) by the MIF method. The antigens B, D, E, G, F, J were prepared from EBs of different *C. trachomatis* serotypes grown in McCoy cells. EBs were isolated from the cells 2 days post-inoculation and purified using conventional techniques (Miyashita & Matsumoto 1992). The serotype EB aliquots were stored in formalin prior to use. The specificity of the antigen preparations was confirmed by *C. trachomatis* serotype-specific monoclonal antibodies. The serotype-specific IgG antibody titres were measured by MIF using the same conjugate and substrate as described above. The serum samples were analysed at twofold dilutions. Titres $\geq 16$ were considered as positive.

In the MIF and in the other serological tests, the antibody determinations of each case and the individual control(s) were always tested simultaneously in the same titration series in a blinded fashion.
5.1.2. Detection of immune complexes (III, IV, V)

ICs were isolated by polyethylene glycol 6000 precipitation (PEG; Fluka, Buchs, Switzerland; Linnanmäki et al. 1993). In brief, 100 µl of the serum sample was added to an equal volume of 7% PEG in sodium borate buffer, pH 8.4, and the mixture was incubated overnight at 4°C followed by centrifugation at 4 500 g for 15 min. The pellets were then washed twice with 3.5% PEG-borate. Finally, the precipitates were dissolved to the original volume in phosphate-buffered saline (PBS). The obtained ICs were analysed by MIF for the presence of *C. pneumoniae*, *C. trachomatis*, and *H. pylori* antibodies. The chlamydial ICs were analysed by MIF for the presence of *C. pneumoniae* and *C. trachomatis* antibodies at twofold dilutions, starting at 1:2. A titre of $\geq 2$ by MIF was considered positive. The antibody and IC determinations of each case and the individual controls were always tested simultaneously in the same titration series in a blinded fashion.

5.1.3. EIAs for chlamydia antibodies (II, V)

Total IgG antibodies and IC bound antibodies to *C. trachomatis* were measured according to the instructions of manufacturers by two commercial EIA kits (Labsystems, Helsinki, Finland). In the EB EIA (I, II, V), twenty EIA units (EIU) was used as the cut-off level of positivity for total IgG antibodies and ICs for *C. trachomatis*. The peptide EIA (II) utilises *C. trachomatis* MOMP variable domain IV synthetic peptide as antigen (Närvänä et al. 1998). An optic density (OD) value >100 was used as a cut-off level for positivity.

For the measurement of IgG antibodies to *C. pneumoniae*, an in-house modification of the EB EIA was used (V). The plates were coated with *C. pneumoniae* Kajaani 6 strain EBs diluted in PBS. The antibodies were tested at dilution of 1:100 and the ICs at dilution of 1:25 in PBS containing 10% fetal bovine serum (FBS; GIBCO BRL, Paisley, Scotland). Alkaline phosphatase-conjugated antihuman IgG (Sigma, St.Louis, MO, USA) was used as the conjugate and 1 mg/ml paranitrophenyl phosphate (Sigma) as the substrate. The antibody levels were expressed as background-corrected EIU based on a known MIF results of positive or negative serum sample (Lehtinen et al. 1989). The cut-off level for *C. pneumoniae* antibody measurement was 80 EIU and for ICs 12.5 EIU, respectively.

5.2. EIA for HPV antibodies (III, IV)

IgG antibodies specific to HPV16, 18 and 33 were determined by EIAs, using capsid antigens, monoclonal mouse anti-human IgG and horseradish peroxidase-labelled goat anti-mouse IgG conjugate (Kirnbauer et al. 1994, Dillner et al. 1996).
5.3. EIA for *H. pylori* antibodies (V)

Serum and IC-bound IgG antibodies to *H. pylori* were tested with a commercial EIA (Pyloriset IgG, Orion Diagnostica, Espoo, Finland). Sera and ICs were diluted 1:200 and 1:50, respectively. According to manufacturer, titres of 300 were used as cut-off levels for positivity both in sera and in ICs.

5.4. EIA for HSV-2 antibodies (IV)

IgG antibodies to HSV-2 were analysed by a commercial EIA (Biokit SA, Spain), using a purified baculovirus-expressed glycoprotein G2 as antigen. According to manufacturer, the OD of the mean ±2 standard deviations of the negative reference sera was used as the cut-off level.

5.5. EIA for pneumolysin antibodies (V)

For the measurement of IC-bound antibodies to pneumolysin, the plates were coated with pneumolysin (5 µg/ml PBS), (kindly provided by Dr. James Paton, Adelaide, Australia) and the ICs (precipitated as described above) were tested at a dilution of 1:50. The same conjugate and substrate were used as described in chapter 5.1.3. OD value over 0.5 was considered to indicate the presence of pneumolysin antibodies in ICs.

5.6. EIA for HL cell antibodies (V)

To determine antibodies to HL cells (cell line for chlamydia culture) the plates were coated with HL cells in a dilution of 100 000 cell/ml in PBS-FBS and the sera were diluted to 1:50 with PBS-FBS. The same conjugate and substrate were used as described in chapter 5.1.3. The sera with OD values over 0.5 were considered to contain antibodies to HL cells.

5.7. Serum cotinine levels (I, II, IV)

Serum cotinine, a surrogate marker of smoking at levels of 20 ng/ml, was analysed by radioimmunoassay using a hapten-specific monoclonal antibody (Parish *et al*. 1995).
5.8. PCR for the detection of *C. trachomatis* (II)

Two or three 10 µm sections of paraffin-embedded biopsy specimens were used for DNA extraction as described (Lie *et al.* 1997). The sections were deparaffinised and the DNA was extracted. The DNA concentration was measured by a spectrophotometer and verified in seventy-nine (93%) of the 85 paraffin-embedded cervical biopsy specimens with human HLA-DQ1 gene by PCR. The presence of *C. trachomatis* DNA was determined from 79 of these specimens by an automatic Cobas Amplicor *C. trachomatis* test (Roche Molecular Diagnostics, Branchburg, New Jersey, USA; Loeffelholz *et al.* 1992). The test uses the primers CP24 and CP27 to define a DNA sequence of 207 nucleotides within the cryptic plasmid of *C. trachomatis*. An internal control has been added to the Cobas Amplicor test to identify processed specimens containing substances that interfere with PCR amplification.

5.9. Statistical analyses

The analysis of cancer risk in relation to the evidence of chlamydial infection was based on keeping the original matched pairs as the units of analysis. The essential case-control pairs are the pairs discordant regarding exposure, *i.e.* the pairs with only the case exposed or only the control(s) exposed. Pairs where both case and control(s) were positive or negative added nothing to our analysis, and were disregarded when estimating relative risks (RR). To estimate RRs associated with evidence of infection, odds ratios [OR with 95% confidence interval (CI)] were calculated using a conditional logistic regression model (Breslow & Day 1980). Exact methods were used when appropriate. The effect of confounding factors were considered by adjusting for relevant variables. The differences in the antibody levels between the cases and controls were tested by the Wilcoxon Signed-Ranks test. Statistical analyses were performed with SPSS, STATA, EGRET, EPIXACT, LogXact and GLIM 3.77 statistical softwares.
6. Results

6.1. *Chlamydia trachomatis* infection as a risk factor for cervical cancer (I, II)

Serum antibodies to *C. trachomatis* were associated with increased risk for cervical carcinoma (unadjusted OR 2.2, 95% CI 1.5-3.3), unlike serum antibodies to *C. pneumoniae* (unadjusted OR 1.2, 95% CI 0.9-1.7). The association remained significant after adjustment for serum cotinine, or serum antibodies to HPV16, 18 and 33 [OR 1.7, 95% CI 1.1-2.7; Table 3 (I)]. No significant difference in the point estimates with regard to the different *C. trachomatis* serovar groups was found after adjustment for cotinine and HPV, and the ORs for the B, C and intermediate groups were 1.6, 2.2 and 1.7, respectively (I).

The cases were then stratified according to the histopathological diagnosis (I). Serum antibodies to *C. trachomatis* were associated with an increased risk for cervical SCC (OR 2.2, 95% CI 1.3-3.5), but not for cervical adenocarcinoma (OR 0.4, 95% CI 0.1-1.7).

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Histology</th>
<th>Unadjusted</th>
<th>Adjusted for any HPV1 &amp; smoking2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>All cervical carcinomas</td>
<td>2.2 (1.5-3.3)</td>
<td>1.7 (1.1-2.7)</td>
</tr>
<tr>
<td></td>
<td>Squamous cell carcinoma</td>
<td>2.8 (1.8-4.4)</td>
<td>2.2 (1.3-3.5)</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>0.5 (0.1-1.8)</td>
<td>0.4 (0.1-1.7)</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>All cervical carcinomas</td>
<td>1.2 (0.9-1.7)</td>
<td>1.2 (0.8-1.8)</td>
</tr>
</tbody>
</table>

1 HPV types 16, 18 and/or 33. 2 Serum cotinine > 20 ng/ml

The ORs for cervical SCC were then analysed after stratification by the stage of the disease (metastatic vs. localised) and the lag between serum sampling and the diagnosis (<60 months, ≥ 60 months) (I). The risk associated with *C. trachomatis* antibodies was higher for metastatic disease (adjusted OR 3.7, 95% CI 1.4-9.4) than for localised disease
A long lag time was associated with an increased risk regardless of age at serum sampling, and the risk increased with the increasing lag time, regardless of the stage of the disease. The distribution of the matched pairs with discordant evidence for infection status indicated that the cervical cancer risk was overall positively associated with past *C. trachomatis* infection, more among subjects aged <37 years than >37 years.

Table 4. Odds ratios (OR) (95% CI) of invasive cervical carcinoma associated with *Chlamydia trachomatis* infection by stage of disease.

<table>
<thead>
<tr>
<th>Stage of disease</th>
<th>Unadjusted</th>
<th>Adjusted for HPVs and smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localised</td>
<td>2.0 (1.1-3.5)</td>
<td>1.6 (0.8-3.0)</td>
</tr>
<tr>
<td>Metastatic</td>
<td>4.6 (1.9-11)</td>
<td>3.7 (1.4-9.4)</td>
</tr>
</tbody>
</table>

1 HPV types 16, 18 and/or 33. 2 Serum cotinine ≥ 20 ng/ml

Serum IgG antibodies to *C. trachomatis* were analysed by three methods (II), *i.e.* by two commercially available EIAs (EB-EIA and peptide-EIA) and MIF. In general, EIA was more sensitive than MIF. EB EIA and peptide EIA both gave equally low point estimates for the risk of cervical carcinoma associated with *C. trachomatis* antibodies (adjusted OR 1.5 and 1.3). Antibodies measured by MIF were associated with higher risk (adjusted OR 1.8, 95% CI 1.1-2.8) showing no major differences in the point estimates with regard to different *C. trachomatis* serotype groups (II).

The highest and highly significant point estimates for the HPV16, HPV18, HPV33 and serum cotinine adjusted risk were found for SCC with more than 12 months’ lag between serum sampling and the cancer diagnosis (II).

Of the single *C. trachomatis* serotypes, serotype G was most strongly associated with the risk of squamous cell carcinoma (adjusted OR 6.6, 95% CI 1.6-27) (Table 5). The other serotypes significantly associated with SCC even after adjustment were I (OR 3.8, 95% CI 1.3-11) and D (OR 2.7, 95% CI 1.3-5.6) (Table 5) (II).

Table 5. Risk for squamous cell cervical carcinoma associated with the presence of IgG antibodies to single *C. trachomatis* serotypes.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Unadjusted</th>
<th>Adjusted for HPVs and serum cotinine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>(0.1-9.6)</td>
</tr>
<tr>
<td>J</td>
<td>3.0</td>
<td>(1.0-9.3)</td>
</tr>
<tr>
<td>H</td>
<td>4.0</td>
<td>(0.9-18)</td>
</tr>
<tr>
<td>I</td>
<td>3.7</td>
<td>(1.4-10)</td>
</tr>
<tr>
<td>G</td>
<td>6.9</td>
<td>(2.1-22)</td>
</tr>
<tr>
<td>F</td>
<td>1.8</td>
<td>(0.5-6.5)</td>
</tr>
<tr>
<td>K</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>4.9</td>
<td>(1.5-17)</td>
</tr>
<tr>
<td>E</td>
<td>2.7</td>
<td>(1.3-5.4)</td>
</tr>
<tr>
<td>D</td>
<td>3.7</td>
<td>(1.9-7.2)</td>
</tr>
</tbody>
</table>

3HPV types 16, 18 and 33
Exposures to more than one different serotypes was associated with increased risk for cervical SCC (p for the trend <0.01; Table 6). *C. trachomatis* DNA was detected only in five (6%) of the 79 cases (II).

Table 6. Risk for cervical squamous cell carcinoma (lag time >12 months) associated with exposure to one or more *C. trachomatis* serotypes.

<table>
<thead>
<tr>
<th>Number of serotypes</th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted for HPVs§ and serum cotinine OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>3.9 (1.6-9.5)</td>
<td>2.2 (0.8-6.1)</td>
</tr>
<tr>
<td>2</td>
<td>5.1 (1.7-15)</td>
<td>6.0 (1.6-23)</td>
</tr>
<tr>
<td>3</td>
<td>6.7 (1.3-35)</td>
<td>4.2 (0.7-26)</td>
</tr>
<tr>
<td>4 or more</td>
<td>4.0 (1.1-14)</td>
<td>4.2 (0.9-19)</td>
</tr>
</tbody>
</table>

§ HPV types 16, 18 and 33

6.2. *Chlamydia pneumoniae* infection as a risk factor for lung cancer (III, IV)

Lung cancer in smoking males (III). Our results showed that the overall lung cancer risk in smoking males was positively associated with chronic *C. pneumoniae* infection. 37% of the cancer cases and 31% of the controls had strong evidence for chronic *C. pneumoniae* infection (IgA ≥ 16 and IC ≥ 4 in both samples), and 16% and 14%, respectively, had moderate evidence (either IgA ≥ 16 in both samples irrespective of IC, or IC ≥ 4 in both samples with IgA ≥ 16 only in the latter sample). The estimated OR, contrasting subjects in the combined category of strong and moderate evidence of infection with those who had no evidence, was 1.6 (95% CI 1.0-2.3) for lung cancer, adjusted for the matching factors, the daily number of cigarettes and the years of smoking. There was no evidence of modification of the relative risk associated with the infection status by daily number of cigarettes and the years of smoking. Antibodies to *C. trachomatis* were not associated with increased risk of lung cancer in smoking males.

When comparing different histological types, a higher relative risk was observed for the combined group of small cell and squamous cell carcinomas (OR 1.7, 95% CI 1.0-2.8) than for the other cancer types (OR 1.3, 95% CI 0.7-2.7).

Stratification by age indicated a particularly high relative risk for all lung cancer types in cases younger than 60 years at recruitment (OR 2.9, 95% CI 1.5-5.4), whereas no association between infection and lung cancer was observed among the older men (OR 0.9, 95% CI 0.5-1.6; p <0.01 for the interaction of age and infection evidence). The detailed ORs according to the strength of the evidence of chronic infection is shown in Table 7.
Table 7. Number of matched pairs of lung cancer cases and control subjects and the adjusted odds ratio (OR) estimates with their 95% confidence intervals (CI), contrasting those with strong or moderate evidence to those with no evidence of chronic \textit{C. pneumoniae} infection.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total number of pairs</th>
<th>OR (95% CI)(^1)</th>
<th>\textit{p}-value for trend(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moderate vs. no evidence</td>
<td>Strong vs. no evidence</td>
<td></td>
</tr>
<tr>
<td>All cancers</td>
<td>230</td>
<td>1.4 (0.8 - 2.5)</td>
<td>1.6 (1.1 - 2.6)</td>
</tr>
<tr>
<td>Small cell and squamous cell carcinomas</td>
<td>154</td>
<td>1.5 (0.7 - 2.9)</td>
<td>1.8 (1.0 - 3.2)</td>
</tr>
<tr>
<td>Other cancers</td>
<td>76</td>
<td>1.3 (0.4 - 4.1)</td>
<td>1.4 (0.7 - 2.8)</td>
</tr>
<tr>
<td>Age &lt; 60 years</td>
<td>103</td>
<td>1.9 (0.8 - 4.4)</td>
<td>3.8 (1.8 - 8.3)</td>
</tr>
<tr>
<td>Age ≥ 60 years</td>
<td>127</td>
<td>1.0 (0.4 - 2.4)</td>
<td>0.9 (0.5 - 1.6)</td>
</tr>
<tr>
<td>Follow-up &lt; 5 years</td>
<td>138</td>
<td>1.2 (0.6- 2.5)</td>
<td>2.0 (1.1 - 3.6)</td>
</tr>
<tr>
<td>Follow-up ≥ 5 years</td>
<td>92</td>
<td>2.2 (0.7 - 6.7)</td>
<td>1.2 (0.5 - 2.5)</td>
</tr>
</tbody>
</table>

\(^1\)adjusted for matching factors (age, study center, treatment group and timing of (samples), daily number of cigarettes and years of regular smoking in conditional logistic regression models.

\(^2\)calculated from a model with quantitative scores (0, 1, 2) for the levels of infection evidence.

\textit{Lung cancer in females (IV).} IgG class antibodies to \textit{C. pneumoniae} were common in Finnish women (66\% among cases, 62\% among controls), whereas IC-bound \textit{C. pneumoniae} antibodies and free serum \textit{C. psittaci} IgG antibodies were rare (10\% and 11\%, and 0\% and 1\%, respectively) (Table 8). No excess risk of lung cancer in females was associated with the presence of free IgA antibodies (7\% and 19\% in cases and controls, respectively) or IC-bound antibodies to \textit{C. pneumoniae} unlike in smoking males in Study III.

In a comparison of the different histological types, \textit{C. pneumoniae} IgG antibodies tended to show a higher, although non-significant, point estimate among squamous cell carcinomas and small cell carcinomas than in other carcinomas (smoking-adjusted RR 1.6, 95\% CI 0.1-21, and RR 0.8, 95\% CI 0.2-2.7, respectively). IgG antibodies to both \textit{C. trachomatis} and HSV-2 were associated with slightly elevated (non-significant) crude risk estimates, but the ORs approached unity after adjustment for smoking. Current smoking was highly significantly (RR= 6.1, 95\% CI 2.3-16) associated with the risk of lung cancer (Table 8).

Table 8. Relative risks, unadjusted and adjusted for smoking, of developing lung cancer associated with serum chlamydial and HSV-2 antibodies in a cohort of 460,000 pregnant women in Finland.

<table>
<thead>
<tr>
<th>Variable</th>
<th>RR</th>
<th>95% CI</th>
<th>Smoking(^1)-adjusted RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking(^1)</td>
<td>6.1</td>
<td>(2.3-16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{C. pneumoniae} IgG</td>
<td>1.1</td>
<td>(0.5-2.7)</td>
<td>0.9 (0.3-2.6)</td>
<td></td>
</tr>
<tr>
<td>\textit{C. pneumoniae} IC</td>
<td>0.9</td>
<td>(0.2-3.7)</td>
<td>0.7 (0.1-3.6)</td>
<td></td>
</tr>
<tr>
<td>\textit{C. psittaci} IgG</td>
<td></td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>0-1</td>
</tr>
<tr>
<td>\textit{C. trachomatis} IgG</td>
<td>1.7</td>
<td>(0.6-4.6)</td>
<td>1.3 (0.4-4.2)</td>
<td></td>
</tr>
<tr>
<td>\textit{C. trachomatis} IC</td>
<td>0.9</td>
<td>(0.2-3.3)</td>
<td>0.4 (0.1-2.3)</td>
<td></td>
</tr>
<tr>
<td>HSV-2 IgG</td>
<td>2.6</td>
<td>(0.5-12)</td>
<td>1.2 (0.2-6.6)</td>
<td></td>
</tr>
<tr>
<td>\textit{C. trachomatis} IgG &amp; HSV-2 IgG</td>
<td>3.8</td>
<td>(0.3-44)</td>
<td>5.4 (0.3-87)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Serum cotinine ≥ 20 ng/ml.
6.3. Chlamydial infections and malignant lymphomas (V)

EIA method seemed to be more sensitive than MIF method for detecting chlamydia IgG antibodies and ICs. Two thirds (67%) of the lymphoma cases and 75% of the controls had IgG antibodies to *C. pneumoniae* in their sera as determined by MIF. Using EIA, IgG antibodies to *C. pneumoniae* were detected in 68% of the cases and 87% of the controls. MIF and EIA revealed antibodies to *C. trachomatis* in 10% and 42% of the cases and 6% and 22% of the controls, respectively. No differences in the prevalence of IgG antibodies to *H. pylori* were found between the cases (40%) and controls (44%). IgA antibodies to *C. pneumoniae* were found in 19% of the cases and 29% of the controls. Three patients and two controls had antibodies to HL cells in their sera, and pneumolysin-specific ICs were detected in only one patient.

*C. pneumoniae*-specific ICs were found in 63% of the cases and 21% of the controls using EIA. *C. trachomatis*-specific ICs were detected in 20% of the cases and 2% of the controls.

The associated overall risk of lymphoma was approximately 10-fold for both *C. pneumoniae* and *C. trachomatis* (OR 9.7, 95% CI 2.9-32 and OR 12.0, 95% CI 1.6-92, respectively) (Table 9). The presence of *C. pneumoniae*-specific ICs and *C. trachomatis* antibodies measured by EIA was associated especially with an increased risk of non-Hodgkin’s lymphoma (Table 9). This association was independent of the clinical aggressiveness of the non-Hodgkin’s lymphoma subgroups. Both chlamydial ICs and *C. trachomatis* antibodies measured by EIA, increased the risk but using MIF method only *C. pneumoniae* ICs affected the risk (Table 9).
Table 9. Odds ratios (OR) and 95% confidence interval (95% CI) of non-Hodgkin’s lymphoma and Hodgkin’s disease according to the presence of Chlamydia and Helicobacter pylori specific IgG antibodies, measured by MIF or EIA.

<table>
<thead>
<tr>
<th>Method</th>
<th>Hodgkin’s disease (n = 19*)</th>
<th>Non-Hodgkin’s lymphoma (n = 53*)</th>
<th>Lymphomas (n = 72*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95 % CI)</td>
<td>OR (95 % CI)</td>
<td>OR (95 % CI)</td>
</tr>
<tr>
<td><strong>MIF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. pneumoniae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>0.7 (0.1-4.0)</td>
<td>0.4 (0.1-1.4)</td>
<td>0.5 (0.2-1.3)</td>
</tr>
<tr>
<td>IC</td>
<td>2.5 (0.5-13)</td>
<td>2.0 (0.9-4.7)</td>
<td>2.1 (1.0-4.5)</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>0.5 (0.0-5.5)</td>
<td>5.0 (0.6-43)</td>
<td>2.0 (0.5-8.0)</td>
</tr>
<tr>
<td>IC</td>
<td>∞ (0.2- ∞)</td>
<td>∞ (0.4- ∞)</td>
<td>∞ (0.9- ∞)</td>
</tr>
<tr>
<td><strong>EIA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. pneumoniae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>0.3 (0.1-1.7)</td>
<td>0.3 (0.1-0.9)</td>
<td>0.3 (0.1-0.8)</td>
</tr>
<tr>
<td>IC</td>
<td>∞ (1.4- ∞)</td>
<td>7.3 (2.2-25)</td>
<td>9.7 (2.9-32)</td>
</tr>
<tr>
<td>C. trachomatis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>1.7 (0.4-7.0)</td>
<td>2.7 (1.1-6.5)</td>
<td>2.4 (1.1-5.0)</td>
</tr>
<tr>
<td>IC</td>
<td>∞ (1.4- ∞)</td>
<td>5.0 (0.6-43)</td>
<td>12 (1.6-92)</td>
</tr>
<tr>
<td>H. pylori</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>0.8 (0.2-3.4)</td>
<td>0.8 (0.4-1.9)</td>
<td>0.8 (0.4-1.7)</td>
</tr>
<tr>
<td>IC</td>
<td>not applicable</td>
<td>4.0 (0.4-36)</td>
<td>4.0 (0.4-36)</td>
</tr>
</tbody>
</table>

*Due to insufficient sample volume, n may vary in the different categories.

The C. pneumoniae-specific ICs measured by MIF were associated with an increased risk of lymphoma (OR 3.8, 95% CI 1.2-11.3) among males, but not among females (OR 1.0, 95% CI 0.3-3.1) (Table 10). The association was especially found in non-Hodgkin’s lymphoma (OR 6.5 95% CI 1.5-29). This association was independent of the clinical aggressiveness of the non-Hodgkin’s lymphoma subgroups. No association was found between the C. trachomatis-specific ICs measured by MIF. However, an increased risk was revealed in all lymphoma types among males by IC EIA. No significant gender-specific differences were found in H. pylori ICs (Table 10).
Table 10. Odds ratios (OR) and 95% confidence intervals (95% CI) of male and female lymphoma patients according to presence of chlamydia and Helicobacter pylori-specific immune complexes (IC).

<table>
<thead>
<tr>
<th>Immune complexes</th>
<th>Males (n=41)</th>
<th>Females (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>OR</td>
</tr>
<tr>
<td><strong>C. pneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC MIF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphomas</td>
<td>37</td>
<td>3.8</td>
</tr>
<tr>
<td>HD</td>
<td>11</td>
<td>1.0</td>
</tr>
<tr>
<td>NHL</td>
<td>26</td>
<td>6.5</td>
</tr>
<tr>
<td>IC EIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphomas</td>
<td>36</td>
<td>=</td>
</tr>
<tr>
<td>HD</td>
<td>11</td>
<td>=</td>
</tr>
<tr>
<td>NHL</td>
<td>25</td>
<td>=</td>
</tr>
<tr>
<td><strong>C. trachomatis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC MIF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphomas</td>
<td>41</td>
<td>=</td>
</tr>
<tr>
<td>HD</td>
<td>12</td>
<td>=</td>
</tr>
<tr>
<td>NHL</td>
<td>29</td>
<td>=</td>
</tr>
<tr>
<td>IC EIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphomas</td>
<td>35</td>
<td>=</td>
</tr>
<tr>
<td>HD</td>
<td>10</td>
<td>=</td>
</tr>
<tr>
<td>NHL</td>
<td>25</td>
<td>=</td>
</tr>
<tr>
<td><strong>H. pylori</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC EIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphomas</td>
<td>36</td>
<td>=</td>
</tr>
<tr>
<td>HD</td>
<td>11</td>
<td>=</td>
</tr>
<tr>
<td>NHL</td>
<td>25</td>
<td>=</td>
</tr>
</tbody>
</table>

*Due to insufficient sample volume, n may vary in the different categories

**HD = Hodgkin’s disease, NHL = non-Hodgkin’s lymphoma
7. Discussion

7.1. Study populations

The study populations, even if quite different compared to each other, were well-defined and population based suggesting that selection bias was unlikely. Evidence based on longitudinal studies (I-IV) (Wallin 1999) is always stronger than evidence-based on cross-sectional case-control studies (only study V was cross-sectional). Serum banks and cancer registries are ideal for systematic evaluation of exposure to chlamydial infections and identification of individuals who reach the end-point (I, II, IV). The cancer registries used achieve almost 100% coverage. Cancer cases and matched controls were identified by linking the data files of the serum banks and the cancer registries using the unique personal identification numbers.

In all studies, controls were matched for age, sex, place of residence and timing of serum sampling. This is important since the characteristics are age- and sex-dependent. *C. pneumoniae* epidemics induce temporally high titres of antibodies, and the site and time of serum sampling hence had to be identical for the cases and controls. Some residual confounding due to possible associations between other exposures to carcinogenic substances (*e.g.*, radon) and chlamydial infection may still remain, but the role of such confounding is likely to be minimal. On the other hand, the statistical precision of our relative risk point estimates was not very high due to the small numbers of discordant pairs in the study IV.

7.2. Methods

The serological tests used in our studies have been extensively evaluated (Puolakkainen *et al.* 1986, Saikku & Wang 1987, Kuo *et al.* 1995). After infection with *C. trachomatis* or HPV16, IgG antibodies are detectable by MIF or EIA at least 5 to 10 years (Puolakkainen *et al.* 1986, af Geijersstam *et al.* 1998). IgG antibodies to *C. pneumoniae* may be detected for over 3 years after acute infection (Kuo *et al.* 1995). Antibodies and metabolites of low molecular weight are reasonably stable for several years in serum at
-25° (Jellum et al. 1995). Matching of cases and controls by storage time was performed to rule out potential confounding.

MIF is considered the golden standard for the serological diagnosis of chlamydial infections. MIF and EIA tests measure antibodies to partly different structural components of chlamydia. The MIF test by Wang and Grayston (1970) is a specific measure of antibodies exposed to chlamydial surface proteins when done properly, i.e. accepting only specific, evenly distributed particle fluorescence. EIA measures antibodies to both superficial and internal proteins and LPS, and may thus be less specific than MIF. It is possible that in chronic infections antibodies are also formed against conserved, more cross-reactive structures, and EIA may thus be more sensitive than MIF for the detection of chronic infection.

In the IC precipitation, we wanted to exclude the precipitation of free serum antibodies by using several serological controls for both methods (MIF and EIA), to confirm that antibodies measured were really derived from ICs. Furthermore, we also wanted to exclude the possibility that the chlamydial antibodies detected were autoantibodies against tissue antigens present in chlamydial antigen preparations (prepared in tissue cultures). This was done by measuring antibodies to HL-cells in the sera. To confirm the specificity of the antibody findings, we measured antibodies to other chlamydial or microbial antigens: to C. pneumoniae in the studies I and II, to C. trachomatis in the studies III and IV, and to H. pylori and pneumococci in study V. To avoid internal measurement errors, the antibody and IC determinations were always done simultaneously in the same titration series in a blinded fashion.

Tobacco smoking is the most important etiologic factor for lung cancer. Smoking and sexual risk-taking behaviour are risk factors for cervical carcinoma. In the study of male smokers (III) confounding due to tobacco smoking was controlled by restricting the study to smokers only and by adjusting for the daily number of cigarettes and the years of smoking in the logistic regression model. In the studies on cervical cancer (I, II) and female lung cancer (IV), tobacco exposure was measured by cotinine analyses (Parish et al. 1995). Serum cotinine as a marker of current smoking does not reflect smoking history, such as the age when smoking was started or the daily dose. Cotinine measurement reflected the time of serum collection. However, the lack of complete control for past smoking is not likely to account for the observed association between C. trachomatis and cervical carcinoma. The effect of smoking and infection with HPV types 16, 18 or 33 was taken into account by adjusting for serum cotinine and antibodies to HPV types 16, 18 and 33 (I, II). Since smoking is not considered a risk factor for lymphomas, the effect of smoking was not tested in the lymphoma study.

The persistence of elevated antibody titers is generally considered as a sign of chronic infection (Brett et al. 1990, Saikku 1992). After an acute C. pneumoniae infection, IgG antibody titres rise and usually decrease slowly, whereas IgA antibodies tend to disappear rapidly, the half-life of IgA being only 5 to 6 days. In reinfection, the IgA response is often prominent. Elevated IgA titers have been considered as a reliable marker of chronic bacterial infection (Brett et al. 1990, von Hertzen et al. 1995, Saikku 1992). Furthermore, circulating ICs containing microbial components are often observed in chronic infections (Højby et al. 1986, Saikku 1992). ICs consist of components of infecting organism and antibodies produced against them. After the recovery, these are slowly removed from circulation. The consistent presence of microbe-specific ICs is a sign of continuous
production of microbial antigens and a potential marker of persistent infection. Thus, the persistent presence of antibodies and ICs, might indicate which individuals are likely to harbour chlamydia, but do not tell the site of the chronic infection process (Saikku 1999).

The first evidence of an association between *C. pneumoniae* and chronic inflammatory processes was provided by the MIF method (Saikku et al. 1988b). In MIF, the reading conditions must be strictly controlled, cases and controls must be tested simultaneously and the results should be read blindly by the same observer. MIF is a subjective test, and the results depend not only on the observer, but also on the equipment and reagents used. Currently, there is no standardised quality control system for checking the performance of chlamydial MIF performed in different laboratories, but standardisation between laboratories is emerging (Peeling et al. 1998). *C. pneumoniae* antibodies are very common, especially in the older age, and furthermore, epidemics may induce temporarily high antibody titres in control populations resulting in difficulties to serologically prove an association with a specific disease. Also, the site of chronic infection remains unknown. Despite these potential problems, however, persistent high chlamydial antibody titres may reflect continuous production of microbial antigen in the host or be a sign of decreased defence mechanisms against an intracellular pathogen (Saikku 1999).

The most specific diagnosis of chlamydial infections can be made by nucleic acid amplification methods. Some problems are inherent to these methods, such as, how to obtain an adequate sample and how to process it to extract reactive nucleic acids properly. Interfering components must be removed, and strict contamination control is essential. On the other hand, nucleic acid detection is not adequate for tissue samples containing minimal amounts of chlamydia with interfering substances, even when human DNA gives a positive signal. Moreover, the target cells for *C. trachomatis* are endocervical glandular cells (Paavonen et al. 1988), which may not always present in the tissue biopsy. Finally exposure to *C. trachomatis* can take place several years or even decades before the diagnosis of cervical carcinoma (Lehtinen et al. 1996). Therefore, it could be expected that only few tissue specimens were positive for *C. trachomatis* DNA at the time of the diagnosis of carcinoma. However, PCR has already yielded compelling evidence for the presence of the organism in atherosclerotic lesions (Kuo et al. 1993).

### 7.3. Major findings

#### 7.3.1. *Chlamydia trachomatis* infection as a risk factor for cervical cancer

We found that the increased risk associated with *C. trachomatis* antibodies was specific for cervical squamous cell carcinoma, and not for adenocarcinoma. IgG antibodies to *C. trachomatis* serotype G were associated with a highly increased risk for subsequent development of SCC. Antibodies to serotypes I and D were also statistically significantly associated with the risk for SCC. We found that the presence of serum IgG antibodies to more than one serotype of *C. trachomatis*, indicating that exposure to multiple serotypes, increased the risk for SCC.
Sexual activity is a risk factor for cervical carcinoma (Fraumeni et al. 1969, Rotkin 1973). Recent longitudinal studies have confirmed the etiological role of the oncogenic HPV types 16 and 18 in cervical carcinoma (IARC 1995, Lehtinen et al. 1996, Dillner et al. 1997). These studies as well as case-control studies have suggested that exposure to C. trachomatis infection also might be involved (Hakama et al. 1993, Bosch et al. 1996, Lehtinen et al. 1996, Muñoz et al. 1996). It seems, that cervical carcinomas without HPV nucleic acids have worse prognosis than those with HPV infection (Higgins et al. 1991). However, C. trachomatis associated risk for cervical carcinomas in this study was higher for metastatic disease than for localised one. Our study supports earlier studies showing an association between C. trachomatis and cervical neoplasia (Paavonen et al. 1979, Schachter et al. 1982, Kiviat et al. 1985, Hakama et al. 1993, Bosch et al. 1996, Lehtinen et al. 1996).

Chlamydial infection was associated with cervical SCC and not with adenocarcinoma. This association is somewhat surprising, since it is well known that endocervical glandular cells are target cells for C. trachomatis. However, with increasing age, the transformed metaplastic cells in the endocervical epithelium, are also target cells for C. trachomatis. Our parallel studies on other genital cancers using a similar study design and methods indicated that non-cervical anogenital cancers were associated with HPV, but not with C. trachomatis (Bjørge et al. 1997). Taken together, these results suggest that the effect of C. trachomatis is specific for cervical cancer and argues against the possibility of residual confounding by HPV.

Serotype G has been associated with symptomatic infections and upper genital tract infections (Dean et al. 1995, Lan et al. 1995). Thus, specific C. trachomatis serotypes might be more virulent and perhaps less sensitive to appropriate antimicrobial treatment (Dean et al. 1998) and act in tumor development in the same way as some H. pylori strains associated with gastric carcinoma (Blaser et al. 1995, Chow et al. 1998).

Multiple serotypes of C. trachomatis have been detected in women with several sex partners or with upper genital tract infections (Barnes et al. 1985, Saikku & Wang 1987, Lin et al. 1998). The presence of mixed infections implies that infection with one serotype does not induce protective immunity against subsequent infection with another serotype (Barnes et al. 1985). The broadly reactive antigens of C. trachomatis found in chronic infections may result in antibodies formed against conserved cross-reactive epitopes. Therefore, the multiple responses discovered in patients with SCC may also suggest chronic infection by any of the serotypes. At the moment, we cannot distinguish between these two alternatives.

Multiple steps are probably necessary for cervical carcinogenesis, and sexual or reproductive factors, sexually transmitted infections, genetic predisposition and tobacco smoking may all play a part. However, our prospective study provides seroepidemiological evidence that past infection with C. trachomatis, as demonstrated by the presence of species-specific MIF antibodies confers an increased risk associated with cervical squamous cell carcinoma.
7.3.2. *Chlamydia pneumoniae* infection as a risk factor for lung cancer

In Study III, we showed that chronic *C. pneumoniae* infection may be a new independent risk factor for lung cancer. We found that markers suggesting the persistence of *C. pneumoniae* infection (i.e. stable elevated *C. pneumoniae* IgA antibodies and ICs) were associated with lung cancer in male smokers. This association was strongest among the cases with small cell and squamous cell carcinoma and appeared to be limited to young and middle aged men (< 60 years). In Study IV, however, we were not able to find a statistically significant risk for lung cancer in association with chlamydial antibodies among young women.

We also found that chronic *C. pneumoniae* infection was associated with small cell carcinoma and squamous cell carcinoma but not with adenocarcinoma in smoking males. A similar trend was also seen in females (Study IV), but the small number of cases may explain that this association could not be confirmed. Thus, the results do not preclude the possibility that chlamydia could also play a role in lung cancer in women.

Lung cancer in women differs from that in men. Most of the histological and age differences between the genders can be attributed to the different smoking habits and the different effects of smoking (Peto *et al.* 1992, Wynder & Hoffmann, 1994). Because fertile-aged women with cervical carcinoma *in situ* (CIS) or other anogenital cancers have an increased risk for primary lung cancer (Bjørge *et al.* 1995, Frisch & Melbye 1995, Levi *et al.* 1996), *C. trachomatis* may also be involved.

Chronic infections may predispose to malignant growth. Chronic bronchitis and other previous lung diseases are known risk factors for lung cancer, in both smokers and non-smokers (Osann 1991). Recent study confirms our finding that chronic *C. pneumoniae* infection is associated with lung cancer (Koyi *et al.* 1999). Also, a role for *C. psittaci* infection in lung cancer has been suggested (Gardiner *et al.* 1992, Alavanja *et al.* 1996, Modigh *et al.* 1996). The possibility that chronic *C. pneumoniae* infection induces carcinogenesis in lung tissue might be a logical consequence related to chronic antigen exposure.

7.3.3. Chlamydial infections and malignant lymphomas

Lymphoma patients had significantly more often *C. pneumoniae*- and *C. trachomatis*-specific ICs in their circulation than controls. The lymphoma risk was considerably higher in males than in females and especially associated with non-Hodgkin’s lymphoma. Free serum antibodies were more common in the controls. Evidently, the antibodies in patients are bound to ICs and are not measurable free in serum, or their antibody production is defective due to the underlying disease. The phenomenon of males having more *C. pneumoniae* antibodies than females has been previously shown in several studies (Saikku 1992, Karvonen *et al.* 1993, Kuo *et al.* 1995).

*Chlamydiae* are known to be able to multiply in the reticuloendothelial system. *C. pneumoniae* infection is also linked with bronchus associated lymphoma tissue in experimental animals (Kimura 1994) and the LGV biovar of *C. trachomatis* causes lymph
node infection (reviewed by Perine & Stamm 1999). Recent study suggests an association with *C. pneumoniae* infection and cutaneous T-cell lymphoma (Abrams *et al.* 1999). Hence, it is logical that chlamydial infection may be one of the factors in the line involved with lymphomagenesis. Another bacterial pathogen, *H. pylori*, has been associated with gastric B-cell lymphoma as well (Wotherspoon *et al.* 1991).

Our study provides epidemiological evidence to suggest that serological markers of chronic chlamydial infection are associated with higher risk for lymphomas. It is not known whether immunological perturbations associated with lymphomas make the patient more vulnerable for chronic chlamydial infections or whether there is an underlying pathogenetic mechanism to link these two conditions.

### 7.3.4. Is the association between chlamydial infection and cancer causal?

Koch’s postulates (1882) are used in assessing the causality of a microbial agent and a disease. According to Koch’s postulates 1) the agent should be detected in all disease cases; 2) the agent should be isolated and grown in laboratory; 3) the laboratory-grown agent should cause disease in animals and 4) the agent should be reisolated from diseased animals. To prove chlamydia as a causative agent in malignant condition is almost impossible by using Koch’s postulates. Chlamydia are difficult to isolate and grow in the laboratory, and experimental animal models of cancer are lacking. Chlamydia have been shown to cause not only one disease, but several clinical conditions (Kuo *et al.* 1995, Stamm 1999). Moreover, most cancers probably are multifactorial. So, chlamydia cannot be expected to be found in most cases. On the other hand, Koch’s postulates cannot be met for most chronic infections, and not even for some acute infections (Fredricks & Relman 1996).

However, other criteria have been suggested in assessing causality. The strength of an association, evidence of dose response and the consistency and specificity of the findings, temporal relation and biological plausibility of a causal association have been proposed as new main criteria by Hill (1965).

In general, the risk of cancer increases with age. We showed that chlamydia-associated risk for cancer was higher among younger patients and the risk seemed to decrease among older patients. This fortifies the strength of the association between chlamydia and lung cancer. Dose response is difficult to analyse in these kind of studies. However, we found that strong evidence of chronic infection, i.e. higher chlamydial serum antibodies, increased the risk of lung cancer more than moderate evidence of chronic infection.

Consistency of the association refers to the need for similar findings by different investigators and by different techniques. *C. trachomatis* has been previously shown to be associated with cervical cancer (Hakama *et al.* 1993, Lehtinen *et al.* 1996), but lung cancer or lymphomas have not been studied earlier. We were not able to confirm the association between *C. pneumoniae* and lung cancer among women, but a Swedish group found an association between *C. pneumoniae* antibodies and lung cancer (Koyi *et al.* 1999). Furthermore, recent evidence demonstrate that *C. pneumoniae* is associated with cutaneous T-cell lymphoma (Abrams *et al.* 1999). Also, the fact that *C. trachomatis* is
associated with cervical carcinoma, and whereas *C. pneumoniae* with lung cancer suggests that the association may be specific.

Our results showed that chlamydial infection precedes the onset of cancer. *C. trachomatis*-associated risk of squamous cell cervical carcinoma increased with increasing time lag from serum sampling to cancer diagnosis. This suggests that there is a temporal relationship between chlamydial infection and malignancy. In the lung cancer study, however, the time between serum sampling and clinical manifestation of the cancer was relatively short (i.e. 3-8 years). Within this time range we could not find any significant difference in the relative risk between longer and shorter follow-up. Thus, it is possible that the higher prevalence of antibodies among cancer patients may be caused by activation of infection by a latent cancer. Several prospective studies have shown that persons with higher concentration of serum antibodies against microbial antigens have increased risk of subsequent development of malignancy.

Infection is invariably accompanied by formation of reactive oxygen and nitrogen species that can damage DNA, proteins and cell membranes (Oshima & Bartsch 1994, Rosin *et al.* 1994, Parsonnet 1999). Inflammation induce increasing cell damage, cell death and compensatory cell proliferation. This process and its influence may lead to mutagenesis (Parsonnet 1999, Rosin & Hofseth 1999). DNA damage caused by oxidative stress resulting from chlamydial infection (Mayer *et al.* 1993), may be one mechanism for chlamydia-induced carcinogenesis.

Apoptosis, programmed cell death, is an active process which limits the accumulation of potentially harmful cells, such as virus-infected cells and tumour cells (Reed 1995). Host cells can respond to intracellular bacterial invasion with apoptosis (Finlay & Cossart 1997, Zychlinsky & Sansonetti 1997). However, chlamydia has an antiapoptotic activity (Fan *et al.* 1998). To obligate intracellular microbes as chlamydia, it is advantageous to inhibit host cell apoptosis in order to secure the intracellular growth cycle and subsequently set favourable conditions for persistent chlamydial infection (Fan *et al.* 1998). However, a failure in an appropriate balance between cell death and cell division may lead to unlimited cell proliferation and neoplasia. In chronic chlamydial infection, this process can initiate or promote carcinogenesis.

Although the biological basis for the relationship between chlamydial infection and malignancy is not yet clear, proposed biological mechanisms and the association of serum chlamydia antibodies and cancer suggest a role for chlamydia in carcinogenesis.
8. Conclusion

We showed that the presence of *C. trachomatis* antibodies is a risk factor for subsequent development of invasive cervical carcinoma and especially for squamous cell carcinoma. We also showed that IgG antibodies to *C. trachomatis* serotype G were more strongly associated with an increased risk of squamous cell cervical carcinoma than other serotypes suggesting that certain serotypes might be more virulent and possibly less sensitive to appropriate antimicrobial treatment. In addition, the presence of serum IgG antibodies to more than one serotype of *C. trachomatis*, indicating exposure to multiple serotypes, further increased the risk of cervical squamous cell carcinoma.

We also showed that the markers of chronic *C. pneumoniae* infection, were associated with small cell and squamous cell lung carcinomas but not with adenocarcinoma in smoking males. A similar trend was also seen in females, although the association was not significant. These findings suggest that chronic *C. pneumoniae* infection may be an independent risk factor for lung cancer in males. The results do not exclude the possibility that chlamydia could also play a role in female lung cancer. Interestingly, in both lung cancer and cervical carcinoma, the increased cancer risk was associated with squamous cell carcinoma, but not with adenocarcinoma.

We also found circulating ICs containing chlamydial antibodies in patients with non-Hodgkin’s lymphomas, suggesting the presence of chlamydial infection in the vascular system. Whether this is a reflection of the altered immune status of these patients or whether chronic chlamydial infections may lead to a connection similar to that between *H. pylori* and mucosa-associated lymphoid tissue, remains to be seen.

In conclusion, these findings show a seroepidemiological association between chlamydial infection and cancer, but do not confirm the causal relationship. Future studies are needed to elucidate the pathogenetic mechanism involved.
9. References


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