Chlamydia pneumoniae in Children - Epidemiology and Clinical Implications

BY

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Abstract

*Chlamydia pneumoniae* is a human respiratory tract pathogen. Seroepidemiological studies indicate that *C. pneumoniae* infection is most common in school-aged children and infrequently detected in younger children.

The aims of this study were to further elucidate the prevalence of *C. pneumoniae* in paediatric populations and to describe the clinical implications of these infections.

The study population consisted of 367 children with respiratory tract diseases, 453 presumed healthy children at day-care, 69 children undergoing adenoectomy and 1585 children from a population based cohort. Family members to infected day-care children were investigated. The laboratory methods used were polymerase chain reaction (PCR) on specimen from upper respiratory tract, serology by microimmunofluorescence (MIF), and immunohistochemistry (IHC) on adenoid tissue specimen. Personal data and medical history were obtained by the means of questionnaires and by the study of patient records.

In children younger than five years, the prevalence of *C. pneumoniae* was 17% as detected by PCR. This prevalence started to increase with increasing age from two years of age. The corresponding increase in serology as detected by MIF started at the age of four years. The prevalence at day-care centres varied from 4 to 39%. Both PCR and MIF underestimated the prevalence of *C. pneumoniae* detected by IHC. Families to infected children were investigated: mothers were more often infected than fathers were.

Most *C. pneumoniae* infections in small children were confined to the upper respiratory tract. These infections were usually mild or asymptomatic. Symptomatic disease may be of prolonged nature. No subsequent illness after *C. pneumoniae* infection was detected at follow-up after four years. In general, no association between *C. pneumoniae* and asthma was found, but *C. pneumoniae* may be of importance for asthma in some susceptible individuals.

Previous *C. pneumoniae* infection reduced the risk for later atopy.

In conclusion, *C. pneumoniae* is a common finding in small children and most often causes relatively mild disease. If the acquisition of this infection early in life will have any implications for future health remains to be investigated.

Keywords: allergy, asthma, children, Chlamydia pneumoniae, immunohistochemistry, microimmunofluorescence, polymerase chain reaction, respiratory tract infection

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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


IV. Normann, E., Gnarpe, J., Gnarpe, H., Wettergren, B. Chlamydia pneumoniae infection predicts a reduced risk for later atopy. (submitted)


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## Abbreviations

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<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EB</td>
<td>Elementary body</td>
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<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>INF-γ</td>
<td>Interferon γ</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>MIF</td>
<td>Microimmunofluorescence</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RB</td>
<td>Reticulate body</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper lymphocyte, type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper lymphocyte, type 2</td>
</tr>
<tr>
<td>TWAR</td>
<td>Taiwan Acute Respiratory (former name for <em>C. pneumoniae</em>)</td>
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INTRODUCTION

*Chlamydia pneumoniae* is a newly identified chlamydial species with unique properties. It is a human pathogen and a common cause of respiratory tract infections. The aim of this study is to describe the epidemiology of the organism in children and to describe the clinical implications of infections in paediatric populations.

History

*Chlamydia pneumoniae* was isolated for the first time in 1965. The organism obtained from the conjunctiva of a child participating in a trachoma vaccine trial in Taiwan was labeled TW-183 and was put in storage for later study (Woolridge 1966, Grayston 1986). Three years later another isolate was acquired in Iran (IOL-207) which was later found to be the same agent (Dwyer 1972). Despite the conjunctival source of these two isolates, serological studies suggested that the organism was not related to eye disease. In 1983, the bacterium was isolated from the respiratory tract for the first time, and designated AR-39 (Grayston 1986). The morphology of the inclusions in cultured cells led to the supposition that the bacterium was an atypical strain of *Chlamydia psittaci* and it was called TWAR (the letters originating from the isolates TW-183 and AR-39). The organism’s role as a human pathogen became apparent in 1985, when it was found to be associated with a mild form of pneumonia (Saikku 1985). After DNA homology studies (Campbell 1987) and ultrastructural analyses (Chi 1987), *Chlamydia pneumoniae* was defined as its own species of *Chlamydia* in 1989 (Grayston 1989a).

Results obtained from analyses of stored sera prove that infections with *C. pneumoniae* were as common in 1958 (Karvonen 1992) and 1963 (Grayston 1992) as they are today.

Taxonomy

When the work with this thesis started, the order *Chlamydiales* had one recognised family, *Chlamydiaceae*, and one single genus, *Chlamydia*, which consisted of four species. There were two human pathogens: *Chlamydia*
trachomatis (Martin 1990, Taylor 1990) and C. pneumoniae; one cattle pathogen named Chlamydia pecorum (Fukushi 1992, 1993); and Chlamydia psittaci (Gregory 1997) which infects birds and animals but also humans.

In 1999, Everett et al. suggested a revision for the taxonomy of the Chlamydiales. Based on DNA homology, they suggested an amended description with four families; the first of these families, the Chlamydiaceae, was divided into two genera: Chlamydia and Chlamydophila. Chlamydia trachomatis remained within genus Chlamydia but the proposal necessitated transfer of the two species C. pneumoniae and C. psittaci to genus Chlamydophila; thus, Chlamydia pneumoniae was to be renamed as Chlamydophila pneumoniae comb. nov (Everett 1999).

Currently, the name Chlamydia pneumoniae is still used in the majority of scientific publications and consequently used in the present thesis (Schachter 2001).

Microbiology

Chlamydial organisms have a unique biphasic life cycle with two distinct forms separated by morphology and functionality. The infectious form, the elementary body (EB) is metabolically inactive and specialised to survive in extracellular environment. The intracellular form, the reticulate body (RB) is metabolically active and capable of reproduction. Chlamydiae are considered as energy parasites because they require adenosine triphosphate (ATP) and nutrient resources from a host cell for metabolism and replication (Moulder 1991, Peeling 1996).

The infective EB of C. pneumoniae is 300–400 nm in size and has by some researchers been found to have a pear-shaped form; this morphology is not seen in other Chlamydiae. EB attaches to and enters the host cells by mechanisms not yet fully understood. The EBs are internalised by active or passive mechanisms dependent on the host cell type. Chlamydiae may enter cells that are not actively phagocytic. The host cell is invaginated and the EB then resides in a membrane-bound vacuole that can evade intracellular defence mechanisms. Host cells can be multiply infected with several EBs and thus theoretically give rise to an even greater number of progeny EBs. Well inside the host, the EB starts to transform into a RB. In culture this first stage is completed within minutes.

When the EB transforms into RB several changes occur in the cell wall of the bacterium and infectivity is lost. The volume of the RB is about 10–100 times that of an EB. Once mature RBs have appeared, multiplication occurs by binary fission. The phagocytic membrane surrounding the ingested EB becomes an inclusion membrane enclosing a colony of dividing RBs. Chlamydiae are incapable of de novo nucleotide synthesis and are dependent on host’s nucleotide pool. Chlamydia pneumoniae requires more host amino
acids than many other chlamydial strains. The yield of new infectious units per host cell ranges from less than 100 to more than 1000, thus the equivalent of 8 to 12 doublings of a single EB. The duration of the growth cycle in culture is about 48–72 hours. Some RBs convert early to EBs whereas others continue to divide until the end of the cycle. At the end the inclusion contains RBs, mature EBs, and intermediate forms.

The developmental cycle completes with the release of chlamydiae from the host cell. In *C. trachomatis* this usually includes lysis of the inclusion followed by lysis of the host cell, however the process may be different with *C. pneumoniae*. Releases of whole inclusions have been seen, as have inclusions extruded by a process resembling exocytosis and thereby sparing the host cell. Only EBs survive outside the host cell.

In addition to respiratory epithelial cells, *C. pneumoniae* infects macrophages (Kaukoranta-Tolvanen 1996, Redecke 1998), endothelial cells (Molestina 1999) and smooth muscle cells (Rödel 2001).

Laboratory methods

Culture is the general “gold-standard” for demonstration of bacteria. *Chlamydia pneumoniae* is an obligate intracellular bacterium and must be cultivated within a eukaryotic host cell.

Culture

Culture provides evidence for the presence of viable organisms. For *C. pneumoniae*, modern methods of culture involve inoculation of a specimen onto a monolayer of cultured cells followed by centrifugation to improve cell to cell contact. The cell lines most often used are originally obtained from human cancers. After incubation of the culture at 37°C for 2–3 days, the cells are stained with a fluorescent-labelled antibody specific for *C. pneumoniae* to visualise the bacteria (Dowell 2001). Unfortunately, *C. pneumoniae* has proved to be slow and difficult to culture and the sensitivity is highly dependent on proper conditions, which includes specimen collection, transport and processing as well as correct culture procedure.

Polymerase chain reaction

The polymerase chain reaction (PCR) is a nucleic acid detection method. The method is rapid, very sensitive and the time used for transportation of specimen to laboratory is not as critical as for culture. PCR does not depend on replicating cells, but instead amplifies a specific nucleotide sequence and the resultant product is then visualised by different techniques. PCR is easily contaminated by extraneous DNA from aerosols and requires strict
adherence to the use of separate rooms for different parts of the process. Negative control specimens must be included in the test runs to rule out contamination. The presence of inhibitors to the Taq polymerase enzyme is a major concern when testing biological specimens. Appropriate positive controls must also be included in every run.

No standardised method for PCR analysis of *C. pneumoniae* has yet been agreed on (Dowell 2001). Various DNA segments specific for *C. pneumoniae* have been used as primers. There are also several different techniques for PCR; some are more validated than others are.

The method used for extraction of *C. pneumoniae* DNA from clinical samples is crucial (Gnarpe 1995). It must vary for different tissue samples to yield as much DNA as possible and it must handle the problem with polymerase inhibitors.

Serology

Serological tests are dependent on the immunological response of an infected host and may be used as evidence for the presence of infectious agents. There is a delay in time between infection and detectable immune response. For patients with primary *C. pneumoniae* infection, IgM appears 2–3 weeks after onset of illness and is generally undetectable after 2–6 months. IgG antibody may not reach high titre until 6–8 weeks after onset of illness. After infection, the IgG titre declines slowly over time. In cases of re-infection, IgM antibody may not appear and the level of IgG may increase quickly, within 1–2 weeks (Kuo 1995). IgA antibodies seem to be produced in reinfection and perhaps chronic infections (Saikku 1992), although the latter may present the same biological situation as reinfection.

For detection of antibodies to chlamydial species the microimmunofluorescence test (MIF) is the “gold standard” (Wang 1970, 1986, Grayston 1990, Dowell 2001). By this test, species-specific antibodies of the IgM, IgA or IgG classes may be detected.

The MIF test uses purified EBs from *C. pneumoniae*, *C. trachomatis* and *C. psittaci* fixed onto glass slides as distinct antigen dots. Dilutions of sample sera are placed over the antigen dots and incubated at 37°C to increase reactivity. The slides are then treated with fluorescent anti-human-antibodies for visualisation. Failure to remove IgG before IgM testing may lead to false positive IgM results due to the presence of rheumatoid factor in sera (Ekman 1993). Since the microscopic reading as well as the interpretation of the pattern of fluorescence is difficult, experience with the technique is a prerequisite for consistent results.

Diagnostic criteria have not been standardised but some recommendations exist for: IgM≥16 or a four-fold increase of the IgG titre in a convalescent serum specimen indicate acute infection. Any IgG titre may denote past infection. Formerly, IgG≥512 was considered as indicative for acute
infection but is today considered as indicative for possible acute infection. There is no general recommendation for interpretation of IgA titres. The aforementioned criteria have been established for adults and the overall pattern of antibody response in children has not been elucidated (Dowell 2001).

Immunohistochemistry

The immunohistochemical procedure (IHC) is based on staining methods used on tissues labelled with a monoclonal antibody specific for *C. pneumoniae*. The method preserves the tissue morphology and permits localisation of the infectious agent to specific areas and cells.

Epidemiology

Much of the information on the epidemiology of *C. pneumoniae* is based on seroepidemiological studies. Population based investigations have revealed that *C. pneumoniae* is a very common and widespread pathogen. Worldwide, more than 50% of the adult population have antibodies to *C. pneumoniae* (Forsey 1986, Kanamoto 1991, Aldous 1992, Karvonen 1993, Paltiel 1995). Most studies show a higher prevalence of 10% in men as compared with women (Grayston 1992, Karvonen 1993, Paltiel 1995). Since the first infection induces a time-limited antibody response, it is supposed that most people are infected and re-infected throughout life (Kuo 1995).

Seasonal changes in the prevalence of antibodies have been reported. Periods of increased incidence lasting for 2 to 3 years have been reported (Grayston 1989b). Between epidemics an endemic situation persists. Epidemics occur in intervals of 4–8 years (Grayston 1990, Mordhorst 1990, Karvonen 1993). There is evidence of two epidemics in the city of Gävle, Sweden, during 1990–1996 (Gnarpe 1999).

When using culture or PCR on respiratory tract specimens from healthy adults, the endemic prevalence is found to be 2–5% (Gnarpe 1991, Hyman 1995).

Epidemiology of *C. pneumoniae* in children

Population based studies in children indicate a low prevalence of antibodies to *C. pneumoniae* in younger age groups (Figure 1). In children younger than five years, the prevalence is 1–3%, in the age group 5 to 9 years the prevalence is 4–10% and in the age group 10 to 15 years, the prevalence is 19–35% (Burney 1984, Aldous 1992). Higher prevalence is found in Sudanese children aged 0.5–11 years where the prevalence of antibodies
ranges from 18 to 27% (Herrmann 1994). No difference between sexes in children is reported.

![Graph](image)

*Figure 1. Prevalence of MIF antibody to *C. pneumoniae* by age among 1057 children in Seattle. Adapted from Grayston 1994.*


The prevalence may change in different studies due to the laboratory method used, definition of diagnostic criteria, time of season and population chosen. With culture or PCR a prevalence of *C. pneumoniae* in healthy control groups is found to be 5–6% (Emre 1994, Falck 1997a). When combining serology with culture and PCR, there is a report of *C. pneumoniae* prevalence of 28% in children with pneumonia (Block 1995).

Transmission

*Chlamydia pneumoniae* may survive on Formica countertops for 30 hours. On hands the survival time is limited to 10–15 minutes (Falsey 1993). There is no evidence for a bird or animal reservoir (Saikku 1985) and the bacterium in not sexually transmitted (Li 1989). It is assumed that *C. pneumoniae* is transmitted from person to person via the respiratory tract (Hyman 1991, Theunissen 1993).
Transmission appears to be relatively inefficient. When contacts of patients with *C. pneumoniae* are investigated, few cases of secondary infections are detected (Aldous 1992, Grayston 1993). However, clusters of infected individuals have been reported among military trainees (Kleemola 1988, Berdal 1992, Csángó 1997), in a boys’ school (Pether 1989), in families (Mordhorst 1992, Blasi 1994) and in a nursing home setting (Troy 1997). In some of these reports, the findings suggest that asymptomatic individuals may be involved in the transfer of the organism. The incubation period for *C. pneumoniae* seems to be about three weeks (Mordhorst 1994). Epidemics may continue for up to six months in clusters of people living in close proximity, indicating a low transmission rate and a long incubation period.

### Clinical implications

*Chlamydia pneumoniae* was first identified as causing pneumonia and bronchitis (Saikku 1985, Grayston 1990). Later several other diseases, as well as asymptomatic infections have been associated with the organism; these conditions may be of both acute and chronic nature.

### Respiratory tract infections

No set of symptoms is characteristic for *C. pneumoniae* infection. Formerly, the following sequence of signs was suggested to indicate *C. pneumoniae* pneumonia: sub-acute onset, initial pharyngitis sometimes with hoarseness, a biphasic pattern with the development of pneumonia or bronchitis with prolonged cough and increased mucus production (Kuo 1995).

The incidence of pneumonia caused by *C. pneumoniae* in children is low. Less than 1% of children in the age group 7 months to 4 years have evidence of acute *C. pneumoniae* infection and in older children, *C. pneumoniae* is found in 6% of children with pneumonia (Grayston 1994, Jantos 1995, Yamada 1995). One author reports a higher incidence of 28% (Block 1995). *Chlamydia pneumoniae* infection causes 10–20% of pneumonia in adult populations (Grayston 1993, Kauppinen 1995). During a *C. pneumoniae* epidemic, the organism may be one of many etiological agents in 43% of the patients (Kauppinen 1995).

There is no report on the incidence of bronchitis in children but 4–25% of adults with acute bronchitis has evidence of *C. pneumoniae* infection (Grayston 1990, Falck 1994).

Almost 80% of those with lower respiratory tract infection caused by *C. pneumoniae* have a sore throat (Grayston 1990) and *C. pneumoniae* is found in 8% of cases with pharyngitis (Huovinen 1989). *Chlamydia pneumoniae* is
found in patients with chronic pharyngitis (Falck 1995) but not tonsillitis (Hone 1994).

*Chlamydia pneumoniae* may be isolated from middle ear aspirates of otitis media (Ogawa 1990, Storgaard 1997, Block 1997). Others have failed to detect evidence of the organism in ear fluid (Goo 1995).

In adults, long-standing chronic respiratory tract infections with *C. pneumoniae* are reported (Hammerschlag 1992, Falck 1995) as well as asymptomatic infections (Gnarpe 1991, Hyman 1991).

Most infections with *C. pneumoniae* are supposed to be asymptomatic or mild upper respiratory tract infections but this has not been studied in detail (Kuo 1995).

**Asthma and allergy**

Asthma is a chronic inflammatory respiratory tract disease with reversible obstruction of the bronchi; this inflammation could theoretically be linked to a preceding or ongoing infection (Hahn 1999, Daian 2000).

The first to find an association between *C. pneumoniae* and asthma was Hahn in 1991 (Hahn 1991). Respiratory tract infections are known to trigger wheezing in asthma and *C. pneumoniae* may be one of the infectious agents frequently found in studies on asthma exacerbation (Emre 1994, Miyashita 1998, Cunningham 1998, Esposito 2000, Gencay 2001). *Chlamydia pneumoniae* specific IgE has been studied in asthma and cystic fibrosis (Emre 1995, 1996). The results are divergent concerning asthma and the immunological relevance of species specific IgE is not clarified (Larsen 1998, Ikezawa 2001). In adults, elevated titre of IgA specific for *Chlamydiae* is found in patients with recently symptomatic asthma and bronchial hyperresponsiveness (Hahn 1996, Björnsson 1996). Higher titres of specific IgA or IgG correlate with severity in asthma (v Hertzen 1999, Black 2000, Gencay 2001). Other authors have not found evidence of an association between asthma and *C. pneumoniae* infection (Cook 1998, Larsen 1998, Mills 2000, Ferrari 2002).

Previously, non-asthmatic persons have been diagnosed as asthmatics after a *C. pneumoniae* infection indicating the possibility that *C. pneumoniae* may not only trigger exacerbation of asthma, but also initiate the chronic inflammation of asthma (Hahn 1998).

There are only few reports on allergy and *C. pneumoniae*. Some of these indicate a protective association between *C. pneumoniae* infection and allergy. In serum more total-IgE and eosinophils are found in children without *C. pneumoniae* infection as compared with children positive in PCR test for *C. pneumoniae* in samples acquired during bronchoscopy (Schmidt 2001). The eosinophilic inflammation induced by ragweed is inhibited in mice first infected with *C. trachomatis*, suggesting that chlamydial infection may confer possible protective properties for the development of allergy.
(Bilenki 2002). On the contrary, a high specific IgG titre is associated with positive skin prick test for atopy (Ferrari 2002).

Other diseases

*Chlamydia pneumoniae* is associated with chronic obstructive pulmonary disease (v Hertzen 1997) and with aggravation of pulmonary function in cystic fibrosis (Emre 1996). High titres with antibodies to *C. pneumoniae* have been associated with lung cancer in a prospective study (Koyi 2001).

*Chlamydia pneumoniae* is linked to several diseases not directly affecting the respiratory tract. Today, several studies are focused on a probable relationship between *C. pneumoniae* infection and the development of arteriosclerosis. The association was first found in a serological study where elevated antibody titres to *C. pneumoniae* were more frequent in patients with chronic coronary heart disease and acute myocardial infarction than in a control group (Saikku 1988b). This finding was later verified in several studies (Grayston 1997). The organism has been found in atherosclerotic lesions as detected by electron microscopy (Shor 1992). Investigations using immunocytochemistry and/or PCR verify this finding (Grayston 1997). The presence of *C. pneumoniae* in the cardiovascular system is further proved by the finding that viable *C. pneumoniae* may be cultivated from abdominal aortic aneurysms (Karlsson 2000). It has been hypothesised that *C. pneumoniae* is only just an “innocent bystander” in cardiovascular diseases and the full pathoetiologic role for *C. pneumoniae* in these diseases remains to be established (Jackson 1997). In animal models, atherosclerotic changes are noted after intranasal injection of *C. pneumoniae* and the organism is detected in the vessel walls (Grayston 1997).


Pathogenesis

*Chlamydia pneumoniae* EBs enters the human host through the respiratory tract and infects respiratory epithelial cells, and alveolar macrophages. It has a marked ciliostatic effect on respiratory epithelium, which may contribute to the development of respiratory tract infections, either with *C. pneumoniae* or with other agents (Shemer-Avni 1995). *Chlamydia pneumoniae* is often found together with *Streptococcus pneumoniae* in co-infections (Kauppinen 1995).

The pathological process is best described for *C. trachomatis*. In cases of trachoma, a scarring disorder of the cornea, different stages of disease have
been described. In areas where trachoma is endemic, early stages of the disease are characterised by mild clinical symptoms usually in younger individuals from whom the organisms are culturable. Older individuals who have been exposed to repeated infections display ocular scarring and fibrosis, corneal trauma and blindness, but *C. trachomatis* is rarely isolated from the diseased tissue in these patients (Beatty 1994). In genital disease, most infections with *C. trachomatis* are asymptomatic but may progress to an inflammatory disease of the fallopian tubes. Several studies indicate a strong association between serological evidence of previous *C. trachomatis* infection and infertility, but the recovery of viable chlamydiae from tubal biopsy specimens is rare.

All *Chlamydiae* may cause persistent infections. Intracellular RBs may enter a non-replicative and non-infectious stage of development and establish a persistent, long-term relationship with the infected cell. This growth stage is called persistence and is characterised as a form of stressed growth, since stress-response proteins such as the heat shock proteins (hsp) are produced in greatly elevated amounts (LaVerda 1999). The causes of this persistence is not fully understood, but host cell activation by immune regulated cytokines and elevated temperature appear to be two physiologically relevant conditions that can trigger persistence. Immune responses to hsp are correlated with disease sequelae in infected humans, for example, scarring trachoma and tubal infertility due to *C. trachomatis* infection (Brunham 1994, Peeling 1997, 1998). High anti-hsp antibody response has also been associated with coronary artery disease (Mahdi 2002). The hsps are highly conserved proteins and exhibit wide cross-reactivity among eukaryotic cells, parasites and bacteria; thus they may induce an autoimmune reaction (Leinonen 1993). A mechanism of antigenic mimicry has been postulated as a link between chlamydial infections and heart disease (Bachmaier 1999) but this finding has not been confirmed.

Another mechanism by which *C. pneumoniae* may mediate the development of chronic infection is by inhibition of apoptosis in infected cells, which protects the organism from being destroyed during the programmed death of the host cell (Fan 1998, Airenne 2002).

The mechanisms by which *C. pneumoniae* may come in contact with blood vessels has been studied. The organism is found in peripheral blood mononuclear cells, indicating that these cells are the vehicles used by the bacteria to disseminate through the body; these may originate from the lungs (Boman 1998).

The scarring sequelae of trachoma are associated with an inadequate Th1-type response to chlamydial antigens (Holland 1993). Increased stimulation of Th2 cytokines may contribute to the development of persistent ocular infection (Holland 1996). Th1 cytokines and in particular IFN-γ is suggested to be important in the eradication of *Chlamydia* (Penttilä 1998, Rottenberg
1999). The antibodies to *C. pneumoniae* detectable with MIF are not protective (Kaukoranta-Tolvanen 1995).
AIMS OF THE THESIS

- to study the prevalence of *C. pneumoniae* in children
- to study the prevalence of *C. pneumoniae* within the families of infected children
- to study the consequences on present health of *C. pneumoniae* infection in children
- to study later outcome in children after previous *C. pneumoniae* infection
- to compare different diagnostic tools for *C. pneumoniae* infection in children
OBJECTIVES AND POPULATIONS

The studies in Papers I–IV were performed on different populations in Gävle, a city with approximately 90,000 inhabitants located on the eastern coast of mid-Sweden. In Paper V, a population who lived in Stockholm, the capital city of Sweden, was investigated.

The Ethical Committee of the Medical Faculty, Uppsala University, Sweden approved the Studies I–IV before they started. All parents gave informed or written consent before inclusion into the study. The ethical committee of Karolinska Institutet, Stockholm, Sweden, approved the study in Paper V.

Paper I

The study was designed to determine the prevalence of C. pneumoniae in children with respiratory tract diseases. A minor objective was to study the clinical findings during a C. pneumoniae infection. Two diagnostic tools for C. pneumoniae infection were also compared.

Children who sought medical attention at the Department of Paediatrics in Gävle for any symptom from the respiratory tract were consecutively enrolled; symptoms were either respiratory tract infection or an acute episode of wheezing. Due to a mandatory referral system, the children were expected to be very young, very ill or to have had a prolonged time period of disease. Both in- and outpatients were included.

Three hundred and sixty-seven children were enrolled from October 1994 to June 1996. There were 231 boys and 136 girls. As expected the mean age was 3.3 years and the median age was 2.0 years, indicating an age distribution that was skewed towards a greater proportion of younger children.

At inclusion into the study a blood sample was obtained for serology. After about eight weeks the family was contacted for a convalescent serum specimen. This was collected from a total of 224 children (84% of those with positive PCR test). At inclusion a specimen was also obtained for PCR analysis, either from the nasopharynx (n=156) or from the posterior wall of the throat (n=182) depending on the preference of the individual nurse collecting the specimens.
The records of each patient were studied to investigate diagnoses, symptoms and laboratory findings, including x-ray if performed.

Paper II

The study was designed to investigate the prevalence of *C. pneumoniae* in healthy children.

The subjects for the study were found at nine day-care centres. During 1996, four hundred fifty-three children were investigated for evidence of *C. pneumoniae* with a throat swab for PCR analysis. A questionnaire was completed for each child by the parents. The study population comprised 83% of the registered children. The reason for exclusion was not studied; all children present the days of investigation participated. The mean age of the participants was 3.4 years.

Children with evidence of *C. pneumoniae* infection were invited for a clinical examination at the Department of Paediatrics. Ninety-seven PCR positive children responded which included 94 % of all that tested positive. The family members of a positive child were invited for a study of transmission within the family by PCR from throat swabs; 95 mothers, 82 fathers and 102 siblings were studied. A serum sample was obtained for MIF from the study child. There was approximately seven weeks between the PCR and the MIF tests.

Paper III

Different methodologies used for *C. pneumoniae* detection in children yields disparate results and questions are raised regarding the true colonisation rate in children. The study in Paper III was conducted to investigate the prevalence of *C. pneumoniae* in adenoid tissues by the use of three different diagnostic tools.

From April to November 1998, sixty-nine patients listed for elective adenoidectomy were consecutively enrolled into the study. The children were all healthy enough for an elective surgical procedure but had clinical problems involving the upper respiratory tract. The indications for operation were recurrent acute otitis media (n=3), otitis media with effusion (n=16) and/or nasal obstruction (n=63). The adenoid tissue was analysed with IHC. A throat swab for PCR and a blood sample for serology were obtained while under general anaesthetics. A questionnaire regarding health was completed for the study of any clinical correlation with laboratory result.
Paper IV

To investigate long-term consequences of *C. pneumoniae* infection, the same population as used in Paper II was again contacted in 2000, four years after the original study. They were asked to complete a new questionnaire that concerned environmental factors and illnesses during the time period between the two studies.

Families that did not respond to the first request were reminded once. A total of 374 responded to the questionnaire, which includes 83% of the original study population. The children were on the average 7.7 years (range 5–10 y).

Paper V

Asthma and atopy may develop during childhood. This study was designed to investigate a proposed association between these diseases and *C. pneumoniae* infection in young children.

The population consisted of a cohort of children followed prospectively from birth to study the development of atopic disorders in relation with environmental factors. The participating 4098 children were born between February 1994 and November 1996 and lived in predefined areas of Stockholm, Sweden. Access to the community population register ensured that all infants were approached for participation. Actively excluded patients were: families planning to move, families with a seriously ill child, families with insufficient knowledge of Swedish and families already having a child included in the study.

The information used in Paper V was obtained when the children had reached the age of four years. At that time data had been collected by the means of questionnaires at the age of two months, and one, two and four years. At the age of four years, a blood sample was collected from cooperating children and 1585 frozen serum samples were available and could be used in the present study. Sera were also investigated for specific IgE for atopic sensitisation.
LABORATORY METHODS

Polymerase chain reaction (PCR)
Specimens from nasopharynx or the posterior wall of oropharynx were obtained with CTA swabs (*Chlamydia trachomatis* aluminium, BioHospital AB, Kopparberg, Sweden) and immersed in 2SP (sucrose-phosphate buffer). The samples were frozen until preparation at the laboratory.

Specimen preparation was done according to methods described by Gnarpe et al. (Gnarpe 1995). After centrifugation, the supernatant was removed and the pellet was treated with the Amplicor respiratory sample preparation kit (Roche Diagnostic Systems Inc., Branchbury, NJ, USA). By this method the specimen was washed with TRIS buffer with added Triton, then again centrifuged. After removal of the supernatant, NaOH was added to the pellet for heat-assisted lysis of the potentially infected cells. As a final step, TRIS buffer was added for neutralisation.

The PCR was carried out according to Campbell et al. (Campbell 1992) using the primer pair HL-1 and HR-1 which resulted in a 437 base-pair amplification product. The PCR products were visualised in 1-% agarose gel with added ethidium-bromide on an ultraviolet transilluminator after electrophoresis.

According to good laboratory practice, preparation of mixtures, sample preparation and detection of amplification products were performed in separate rooms.

Serology – microimmunofluorescence (MIF)
The sera were frozen at −20°C until tested. Serological analysis was done using the microimmunofluorescence technique (Wang 1970), modified for analysis of *C. pneumoniae*. In Study I and II, slides from I.O. International Ltd., London U.K. were used containing wells for *C. psittaci* (IOL-395), *C. trachomatis* (serovars D-K) and *C. pneumoniae* (IOL-207). In Study III and V the slides were purchased from LabSystems OY, Helsinki, Finland.

A serum dilution of 1/16 was used to screen for IgG, IgA and IgM antibodies in Paper I–III. In Study V, only sera containing IgG were tested.
for IgA. Sera demonstrating IgA or IgM antibodies were diluted with GullSorb (Gull Laboratories, USA) to remove all IgG antibodies and then re-tested. Sera positive in the screen tests were examined using two-fold dilutions. An extended time of incubation over night was used to optimize the sensitivity of the test (Gnarpe 2000). The slides were then washed and dried before addition of fluorescein isothiocyanate-conjugated anti-human IgG, IgA or IgM (Dakopatts, Denmark). All tests in Papers I–III were read by either of two experienced investigators using a Zeiss UV microscope with x 400 magnification. In Study V, an Olympus UV microscope with a magnification of x 500 was used by the investigator.

Immunohistochemistry (IHC)

Tissues were immediately immersed in formalin and fixed before being embedded in paraffin. Sections of 3–4 µm thickness were cut from the tissue blocks and mounted on microscope slides.

Prior to staining the samples were deparaffinised by immersion in xylene and hydrated through a series of alcohols with decreasing concentrations. The staining procedure was based on an avidin-biotin-peroxidase method, StreptABComplex/HRP Duet Kit® (DAKO A/S, Glostrup, Denmark) (Nääs 1999). Slides were initially treated with pepsin solution adjusted to pH 2.0 to allow penetration of the antibody into cells. Dr Kenneth Persson, Malmö, Sweden, generously donated the monoclonal antibody used. This antibody had earlier been compared with RR-402 (DAKO, Denmark) and found to be identical and thus specific for \textit{C. pneumoniae}. After treatment of the tissue with the monoclonal antibody, the sections were covered with biotinylated goat anti-mouse/rabbit antibodies and incubated. After washing, the tissues were covered with ABComplex/HRP and after incubation covered with AEC Substrate System (K0697, DAKO Corp., Carpinterie, CA, USA). For counterstaining, slides were placed in a cuvette filled with Mayer’s hematoxylin (Histolab Products AB, Göteborg, Sweden).

To eliminate the possibility of unspecific staining reactions, the procedure was also tested with a \textit{Bartonella hensalae} monoclonal antibody, kindly donated by Professor L. Engstrand, SMI, Stockholm, Sweden. Negative control on the staining procedure were the same procedure on the same tissue just replacing the monoclonal antibody with PBS (phosphate-buffered saline, pH 7.4).
RESULTS

Epidemiology

Serology
The prevalence of antibody to *C. pneumoniae* in the serological investigations done by MIF is summarised in Table 1. To avoid interference of maternal antibodies children younger than six months are excluded from the tables.

Table 1. Antibodies to *C. pneumoniae* in children. Results extracted from the study papers.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>0.5 – 2</td>
<td>16/179 (9)</td>
<td>9/102 (9)</td>
<td>2/18 (11)</td>
<td>1/13 (8)</td>
<td></td>
</tr>
<tr>
<td>3 – 4</td>
<td>6/65 (9)</td>
<td>6/40 (15)</td>
<td>15/57 (26)</td>
<td>0/21 (0)</td>
<td>161/1585 (10)</td>
</tr>
<tr>
<td>5 – 9</td>
<td>16/44 (36)</td>
<td>14/33 (42)</td>
<td>10/22 (45)</td>
<td>10/27 (37)</td>
<td></td>
</tr>
<tr>
<td>10 – 16</td>
<td>22/39 (56)</td>
<td>18/29 (62)</td>
<td>2/8 (2)</td>
<td></td>
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</tbody>
</table>

Numbers within parenthesis are percent.
Paper I:c: convalescent serum specimen.

In Paper I, the only investigation with an acute and a convalescent serum specimen, there was a possibility to observe a change in antibody titre. Only 12 children of 224 with a convalescent serum specimen (5%) had a four-fold increase in IgG, 3 of these children were younger than two years and 2 in the age group 2–4 years. There were twin girls aged 12 months that seroconverted to 1/1024 and 1/4096, respectively from a titre of <1/16. At the time of titre rise these girls were infected with respiratory syncytial virus.
Table 2. *Anti*-C. pneumoniae-IgG titres of 1/512 or more in children. Results extracted from study papers.

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Paper I*</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper V</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 – 2</td>
<td>6/179 (3)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3 – 4</td>
<td>3/65 (5)</td>
<td>5/57 (9)</td>
<td>0</td>
<td>39/1585 (2)</td>
</tr>
<tr>
<td>5 – 9</td>
<td>8/44 (18)</td>
<td>3/22 (14)</td>
<td>4/27 (15)</td>
<td></td>
</tr>
<tr>
<td>10 – 16</td>
<td>9/39 (23)</td>
<td>2/8 (25)</td>
<td></td>
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</tr>
</tbody>
</table>

Numbers in parenthesis are percent.
* = including results from both acute and convalescent serum

High IgG titres of 1/512 or more (indicating possible acute infection) were detected according to Table 2.

Anti- *C. pneumoniae*-IgM was detected in 5 children with acute respiratory tract infection in Paper I and in 3 children from Paper II. All of the latter had signs of acute upper respiratory tract infection at the time of swab sampling at day-care but were healthy at the later time of blood sampling. In Papers III and V, there were no findings of specific IgM.

Specific IgA to *C. pneumoniae* was found according to Table 3.

Table 3. IgA antibodies to *C. pneumoniae* in children. Results extracted from study papers.

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Paper I*</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper V</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 – 2</td>
<td>4/179 (2)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3 – 4</td>
<td>1/65 (2)</td>
<td>7/57 (12)</td>
<td>0</td>
<td>42/1585 (3)</td>
</tr>
<tr>
<td>5 – 9</td>
<td>9/44 (20)</td>
<td>5/22 (23)</td>
<td>3/27 (11)</td>
<td></td>
</tr>
<tr>
<td>10 – 16</td>
<td>13/39 (33)</td>
<td>2/8 (25)</td>
<td></td>
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</tbody>
</table>

Numbers in parenthesis are percent.
* = including results from both acute and convalescent serum.

There was no serological difference between sexes in this study. If excluding children younger than six months of age, the prevalence of IgG antibodies was 20% in boys and 19% in girls included in Paper I; the corresponding prevalence in Paper V was 10% for both genders. High titres (≥512) of IgG
were found in 8% of boys and 7% of girls in Paper I and in 2% and 3% respectively in Paper V. IgA antibodies were found in 10% of boys and 6% of girls in Paper I and in 2% and 3% respectively in Paper V.

Polymerase chain reaction

*Chlamydia pneumoniae* was detected by PCR in 57 of 360 children (16%) in Paper I, in 103 of 453 children (23%) in Paper II and in 5 of 69 children (7%) in Paper III. The findings of *C. pneumoniae* in different ages are presented in Table 4.

Table 4. *Chlamydia pneumoniae* detected by PCR in children. Results extracted from study papers.

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 2</td>
<td>22/213 (10)</td>
<td>18/125 (14)</td>
<td>1/13 (8)</td>
</tr>
<tr>
<td>3 – 4</td>
<td>12/64 (19)</td>
<td>59/220 (27)</td>
<td>2/21 (10)</td>
</tr>
<tr>
<td>5 – 9</td>
<td>10/44 (23)</td>
<td>26/108 (24)</td>
<td>1/27 (4)</td>
</tr>
<tr>
<td>10 – 16</td>
<td>13/39 (33)</td>
<td>26/108 (24)</td>
<td>1/8 (13)</td>
</tr>
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</table>

Numbers in parenthesis are percent.

In Paper I, 16% of the boys and 15% of the girls had positive PCR test and in Paper II the corresponding results were 20% and 26%. In Paper II, four of the day-care centres were investigated during spring and five centres during the autumn. In the spring 40 of 217 children (18%) were positive in the PCR test and in the autumn 63 of 236 children (27%) tested positive (p<0.05, chi² test). The prevalence in different day-care centres varied between 4 and 39%.

Immunohistochemistry

The specific staining of patient tissues was used only in Paper III: *C. pneumoniae* was detected by IHC in adenoid tissue from 68 of the 69 participating children. The negative child was a boy aged 5 years. *Chlamydia pneumoniae* was found in macrophage-like cells beneath the squamous epithelial surface and within reactive lymph follicles. The bacteria-containing cells were found at the periphery, but not in the centre of these lymph follicles.
Comparison of laboratory methods

In Paper I serological evidence of acute *C. pneumoniae* infection was defined as IgM>16, IgG>512, IgA>64 or a four-fold increase in IgG titre. Using this definition in children with convalescent serum specimen and a conclusive PCR test, 4 of 96 children (4%) younger than two years, 7 of 63 in the age group of 2–4 years (11%), and 25 of 62 in older children (40%) were positive for *C. pneumoniae* by serology. The corresponding prevalences when using PCR in the same age groups were 10%, 27% and 34%, respectively. In children younger than five years the difference between serology and PCR was statistically significant (7% vs. 17%, p<0.01, chi² test). In older children such a difference could not be detected (40% vs. 34%, p>0.4).

Also in Paper I, two different sample collection sites for PCR specimens were used in different individuals: the nasopharynx and the posterior wall of the oropharynx. The nasopharyngeal location was used for 100 children younger than two years, for 42 children aged 2–4 years and for 14 children aged more than four years. The oropharyngeal site was used for the same age groups in 60, 58 and 64 children, respectively. The method used depended strongly on the age of the child: nasopharyngeal swabs were used predominantly on younger children and throat swabs on older children (p<0.001, chi² test). Depending on what site used, there was a difference in yield of positive PCR findings: the nasopharyngeal swab specimen was positive in 11 of 156 tests (7%) as compared with 44 of 182 throat swab specimen (24%). The difference in yield from the two collection sites was statistically significant when corrected for age group (p<0.002, Mantel-Haenszel stratified analysis). The only child younger than two years positive for *C. pneumoniae* by both MIF and PCR had the specimen collected from the oropharynx, all other seropositive children had the specimen obtained from the nasopharynx.

In Paper II a serum sample was obtained approximately 7 weeks after a positive PCR test. Seventy of 97 children had not developed any detectable antibodies during that time period.

In Paper III, the children were investigated with PCR on throat swab specimen, with serology and with IHC done on adenoid tissue. Of 69 children, *C. pneumoniae* was detected by PCR in 5, antibodies to *C. pneumoniae* were found in 14 and with IHC C. pneumoniae was found in adenoids of 68 children.

Family findings

In Paper II the entire family of the child infected with *C. pneumoniae* was invited to the hospital for examination. Of 97 families investigated, 58 had no additional individual infected, 32 had one family member infected, 6 had
2 persons infected and in one family 3 were infected. Families of PCR negative children were not investigated.

Twenty-one of 95 mothers (22%) were positive in the PCR test for C. pneumoniae, as were 18 of 102 siblings (18%) and 7 of 82 fathers (8%); these differences were statistically significant if taken together (p<0.05, chi² test); only the difference between mother and father was significant when one group of family members were compared to another.

The number of siblings at home had an influence on the prevalence of C. pneumoniae detected by PCR. Ten of 25 children (40%) with three siblings had a positive PCR test, as had 21 of 85 children (25%) with two siblings, 49 of 231 (21%) with one sibling and 23 of 112 (20%) with none.

Forty-two of 103 children (41%) with positive PCR tests in Paper II had a smoker living at home as compared with 114 of 350 children who tested negative (33%). The PCR positive children more commonly had furred pets at home (38 of 103) as compared with 71 of 350 PCR negative children who had a furred pet (37% vs. 20%, p<0.001, chi² test).

In Paper III it was noted that for children older than two years of age, seven of 12 participants (58%) with antibodies to C. pneumoniae had a furry pet at home as compared with 10 of 42 children (24%) without antibodies. This finding may have been confounded by age since older children more often had pets at home.

Clinical findings

Respiratory tract infections

In Paper I the clinical diagnoses of upper respiratory tract infection of presumed viral origin and common cold were designated as nasopharyngitis. Forty-six percent of children positive in PCR test for C. pneumoniae were diagnosed as having nasopharyngitis.

Eight of 56 children (14%) with pneumonia had C. pneumoniae infection; the youngest child was three years old. Chlamydia pneumoniae was found in 4 of 11 children (36%) with pseudocroup, in 3 of 4 children older than four years with bronchitis, in 2 of 37 children (5%) with otitis, and in 26 of 124 children (21%) with nasopharyngitis. Thirty-six of 185 children (19%) diagnosed with upper respiratory tract infections had positive PCR tests as compared with 19 of 178 children (11%) with signs from the lower respiratory tract (p<0.05, chi² test).

At clinical examination, no findings in the nose, ear or throat exclusively distinguished a C. pneumoniae infection; nor was there any diversity regarding fever, fatigue, cough, and wheezing as compared with PCR negative children. Pulmonary rales were noticed in 14 of 55 PCR positive
children (25%) and in 122 of 295 PCR negative children (41%). Nine of 13 PCR positive children (69%) and 15 of 75 of PCR negative children (33%) had normal X-rays. Fewer of the PCR positives were hospitalised (21% vs. 11%). The differences regarding pulmonary rales, X-ray and hospitalisation were all statistically significant at p<0.05 using chi² test.

The number of days with illness before attending hospital was recorded. The children with *C. pneumoniae* infection had on average 21 days with disease as compared with 14 days for those without evidence of *C. pneumoniae* infection. When excluding children found to have other respiratory tract pathogens, e.g. *Bordetella pertussis* or respiratory syncytial virus, the PCR positive children had 24 days with symptoms as compared with 14 days for the PCR negative children. For children aged 2–4 years with nasopharyngitis, the PCR positive children had 25 days of disease and the negative children had 10 days before attending hospital. All of these differences were statistically significant (p<0.05, Kruskal-Wallis one-way analysis of variance by ranks for two groups).

In children at day-care, 35% reported any symptom from the respiratory tract at the time of PCR test; *C. pneumoniae* was present in 36 of these 158 children (23%). However, the organism was equally distributed in the whole study population irregardless of the state of respiratory health. The questionnaire also included any respiratory tract symptoms during the week before the test, but no difference between PCR positive and negative children could be found. Questions were posed regarding the health status of the family, but the answers did not differ between groups.

Sixteen of 97 children (16%) with positive PCR test who were followed up reported no signs of respiratory tract infection during the time period from the week before PCR test until the day of blood sampling. Four of 27 children (15%) with antibodies to *C. pneumoniae* were equally free from symptoms during the same period of time.

In children who underwent adenoidectomy, no sign of acute respiratory tract infection was present or the procedure would have been postponed.

In Paper IV, the children of Paper II were followed up for the analyses of illnesses during the four years that had elapsed after the test at day-care. For this time period the PCR positive and negative children reported no difference in proportions of in-patient care due to respiratory tract infections, in experiences of breathing difficulties and in prevalence of pneumonia and pseudocroup. Thirty of 86 children with positive PCR test had experienced acute otitis media as compared with 134 of the PCR negative children (35% vs. 48%, p<0.05, chi² test). There was no significant difference in the total number of episodes with respiratory tract infections.
Asthma and allergy

In Paper I, 7 of 83 children (8%) with asthma were positive by PCR. Children with a long period of wheezing before attending the hospital more often had antibodies to *C. pneumoniae*; those with specific IgG had symptoms for 28 days and those with specific IgA had been ill for 26 days. The asthmatic children without detectable antibodies were unwell for approximately 15–16 days. One boy in Paper I was followed for an extended period of time. This boy had moderate asthma; at inclusion he was 4 years old and attended the clinic due to wheezing. Serology showed a specific IgG titre of 1/2048 and this titre climbed to 1/8192 two months later. The PCR test was then also positive and the boy was prescribed specific antibiotics. He improved and the IgG titre had decreased on follow up. He was then predominantly healthy for five months until he had two severe episodes of wheezing. The PCR test was again positive and the IgG titre had again risen to 1/8092. He started a new course of antibiotic treatment and improved. Ten months later the IgG titre was 1/512 and the PCR test was negative.

In presumed healthy children at day-care 6% of PCR positive children had asthma as had 3% of the PCR negatives; none of these children had respiratory symptoms at the time of test. Consequently, 6 of 16 children with a history of asthma had evidence of *C. pneumoniae* infection in this study. In the same study population 2% of the PCR positive children had any form of allergy as compared with 6% of those negative for *C. pneumoniae* in PCR test. In Paper II, there were no statistically significant results regarding PCR finding, asthma and allergy. Four of 6 children (67%) with a history of asthma had specific antibodies to *C. pneumoniae*, as had 23 of 91 (25%) of those without asthma; this difference was significant in univariate analysis but the result may have been confounded.

There was no conclusive information on asthma or allergy in Paper III.

Five percent of the PCR positive children responding to the study in Paper IV reported that they had asthma at the time of the original study in Paper II; this prevalence had not changed at the time of follow up. Of the PCR negative children 3% reported asthma at the time of the original study and this number had increased at follow-up to 6%. This finding was tested in a multiple logistic regression model including known confounders involved in the development of asthma. In this model the odds ratio for having asthma at follow-up was 0.27 (95% CI, 0.03–2.35) if the PCR test had been positive for *C. pneumoniae* four years before the study (Paper IV, Table 4). The greatest risk for asthma was found in children with heredity for asthma and a negative PCR test (18% vs. 4%, p<0.002, two-tailed Fisher’s exact test).

Of children included in Paper IV, none of the children positive for *C. pneumoniae* in PCR test reported that they had allergy for furred pets or pollen in the original study. At follow-up the prevalence of allergy in the former PCR positive children was 5%. Among the formerly PCR negative
children, 3% reported the same type of allergy in the original study and 13% in the succeeding study. At follow-up, there were significantly more allergic children in the former PCR negative group as compared with the PCR positive children (13% vs. 5%, p<0.03, chi² test). This finding was tested in a multiple logistic regression model: the odds ratio was 0.12 (95% CI, 0.02–0.95) for allergy at follow-up if the PCR test had been positive four years before the study (Paper IV, Table 5). Children with heredity for allergy but who were negative by PCR for \textit{C. pneumoniae} were at greatest risk for allergy (22% vs. 5%, p<0.001, chi² test).

In Paper V, 36 of the 285 children (13%) with asthma had specific antibodies to \textit{C. pneumoniae}, as had 124 of 1300 children (10%) without asthma. The difference between asthmatics and non-asthmatics was statistically true only for girls: 19 of 118 asthmatic girls (16%) had antibodies as compared with 55 of 631 non-asthmatic girls (9%). For boys the prevalence of specific antibodies was equally distributed among asthmatics and non-asthmatics (10%). Without being statistically significant, the finding of antibodies to \textit{C. pneumoniae} was more common in asthmatic children if they were negative in IgE test for atopy: 14% of non-sensitised asthmatics had antibodies to \textit{C. pneumoniae} as had 10% of sensitised asthmatic children. In a multiple logistic regression model including known risk factors for asthma, the odds ratio for being asthmatic if having antibodies to \textit{C. pneumoniae} was 1.4 (95% CI, 0.9–2.0) (Paper V, Table 2). In the same model, the odds ratio for asthma increased to 3.4 (95% CI, 1.2–9.9) if only IgG titres above 1/512 was considered as positive. Such high titre was found only in 6 children with asthma and 5 of these were girls. If excluding children with specific IgA to \textit{C. pneumoniae}, the odds ratio was 1.6 (95% CI, 1.03–2.6) for asthma when having IgG to \textit{C. pneumoniae} using the same logistic regression model; again the girls conferred most information (Paper V, Table 3).

In the matched case-control study included in Paper V, no statistically significant association between antibodies for \textit{C. pneumoniae} and asthma could be found, including tests for high antibody titres and antibody class.

In the study of atopic diseases, there were two significant findings in Paper V. The prevalence of boys with IgG-antibodies to \textit{C. pneumoniae} was lower in sensitised than in non-sensitised boys (6% vs. 12%, p<0.05, chi² test), and the prevalence of girls with IgA antibodies was lower in sensitised than in non-sensitised girls (11% vs. 43%, p<0.05, chi² test). Sensitised girls more often had antibodies than sensitised boys had (12% vs. 6%, p<0.05, chi² test).
Results in short summary

- The prevalence of children with antibodies to *C. pneumoniae*:
  - children aged 0.5–2 years: 9% (range 8–11%)
  - children aged 3–4 years: 11% (range 0–26%)
  - children aged 5–9 years: 28% (range 34–45%)
  - children aged 10–16 years: 51% (range 25–56%)

- The prevalence of children with *C. pneumoniae* as detected by PCR:
  - children aged 0–2 years: 12% (range 8–14%)
  - children aged 3–4 years: 24% (range 10–27%)
  - children aged 5–9 years: 21% (range 4–24%)
  - children aged 10–16 years: 30% (range 13–33%)

- *Chlamydia pneumoniae* was detected by IHC in 99% of children undergoing adenoidectomy

- *Chlamydia pneumoniae* was more prevalent in children having several siblings

- *Chlamydia pneumoniae* was more prevalent in mothers to infected children than in fathers

- Children infected by *C. pneumoniae* detected by PCR more often had furred pets at home

- Infections with *C. pneumoniae* may be asymptomatic

- Infections with *C. pneumoniae* in children were mostly confined to the upper respiratory tract

- Of children attending hospital because of respiratory tract infections, children infected with *C. pneumoniae* most often had a mild or moderate disease but may have been ill for an extended period of time

- Children infected by *C. pneumoniae* as detected by PCR did not report more subsequent illnesses at follow-up after four years

- It was a negative association between former *C. pneumoniae* infection and later atopy

- There were no strong evidence of an association between *C. pneumoniae* and asthma

- Young children did not develop antibodies at the same rate as they were positive for *C. pneumoniae* by PCR

- Throat swab specimen was superior to nasopharyngeal specimen for the detection of *C. pneumoniae* by PCR

- Both PCR and MIF underestimated the prevalence detected by IHC done on adenoid tissue specimen from children undergoing adenoidectomy
DISCUSSION

When comparing different studies, the desideratum is that the methods used are validated and comparable. In the case of *C. pneumoniae* research, there is a problem with the lack of standardised laboratory methods (Dowell 2001). The “gold-standard” for detection of *C. pneumoniae* infection is MIF. However, the method is technically complex and the interpretation subjective. The strength of the present study is that the same investigators read all MIFs. Diagnostic criteria by MIF are not standardised and this may be a difficult issue, especially in children as will be discussed further below. The PCR test is even more associated with laboratory variation. Several methods and primers are used. The method used in the present study is one of the most validated (Dowell 2001), though the preparation of the specimen is further optimised. The test has been used in several studies and found to be valid (Falck 1997a, 1997b). The IHC method used in this study has been published (Falck 1997b, Karlsson 2000, Engstrand 2001). Viable *C. pneumoniae* has been isolated from specimen positive by this IHC method.

Epidemiology

*Chlamydia pneumoniae* was detected by PCR on specimens from the upper respiratory tract in Papers I, II and III, and the prevalence was 16%, 23%, and 7%, respectively. This prevalence was higher than earlier described from population-based studies (Burney 1984, Forsey 1986, Aldous 1992) and from children with lower respiratory tract infections (Saikku 1988a, v Renterghem 1990, Kanamoto 1991, Ouchi 1994, Herrmann 1994, Jantos 1995, Yamada 1995) using serology.

It has been suggested that *C. pneumoniae* infections are most common in school-aged children and that children younger than five years of age are affected much less frequently (Kuo 1995). These conclusions were based on seroepidemiological studies, which also revealed that the prevalence of antibodies was highest in men (Aldous 1992, Grayston 1992). It was recognised that *C. pneumoniae* infection appears to differ from *Mycoplasma pneumoniae* and many viral respiratory tract infections where mothers and young children have a higher incidence of disease. It was presumed that transmission of *C. pneumoniae* most often occurs outside the households (Grayston 1994).
In children younger than five years of age the prevalence of *C. pneumoniae* was 17% as detected by PCR in Papers I–III taken together. The prevalence of *C. pneumoniae* detected by PCR increased with age as reported in Papers I and II with a rapid increase in prevalence with increasing age over two years (Table 4).

When comparing the PCR results with the serological findings in the present study, the prevalence detected by PCR was higher than the seroprevalence detected by MIF in children younger than five years of age as illustrated in Figure 2.

![Figure 2. *Chlamydia pneumoniae* infection detected by MIF and PCR reported in Table 1–4. The differences between MIF and PCR in the age groups 2–4 years and 10–16 years were statistically significant at *p*<0.001 and *p*<0.05, respectively (chi² tests).](image)

The seroprevalence was approximately 10% in small children and this prevalence started to increase rapidly in children older than four years of age. The increase in prevalence by PCR precedes the increase in prevalence of antibodies to *C. pneumoniae* by approximately 2 years. The increase of antibodies at the age of 4–6 years is a consistent finding in several studies (Grayston 1992, Yamada 1995). Thus, in children older than 10 years the seroprevalence exceeded the prevalence detected by PCR (Figure 1).

The data in Figure 1 were condensed from all papers in the present study, collected from dissimilar populations on different occasions; the result must therefore be interpreted cautiously. In Paper I two methods were tested on the same individuals, using defined criteria for diagnostic serology, the PCR technique detected more *C. pneumoniae* infections than MIF in children younger than five years (17% vs. 7%). In Paper II the blood sample was
collected seven weeks after a positive PCR test and after this period of time a serological response was expected to be detectable; however, most children were seronegative at investigation.

The main explanation for the disparate prevalence detected by PCR and MIF may be accounted for by consideration of the detection method; two different phenomena are tested. PCR demonstrates the presence of a living or dead infectious agent while serology detects an immunological response and therefore relies on the subjects’ capability to produce detectable antibodies when infected and does not necessarily indicate an ongoing infection. The results of the present study indicate that small children do not produce antibodies to the same extent as older individuals. It could be that small children have an immature immune system and decreased ability to produce the humoral response detectable by MIF. It is also possible that *C. pneumoniae* is a poor antigen (Leinonen 1993). It can be speculated that the first *C. pneumoniae* infections in children could most often be superficial infections that may not induce the same immunological response as more invasive, repetitive or prolonged infections. In the study presented in Paper I, a pair of one-year-old twins had very high titres to *C. pneumoniae*. These girls were simultaneously infected with respiratory syncytial virus and it may be that this aggressive respiratory tract infection interacted with *C. pneumoniae* and potentiated the serological response to *C. pneumoniae*.

Presumed healthy children and children with all types of respiratory tract infections are included in this thesis. One explanation for the higher prevalence we found as compared with other studies may be that several investigations have been conducted only on children with lower respiratory tract infections. The reason for that selection of study patients may be that *C. pneumoniae* was first recognised as a cause of pneumonia and bronchitis. Most of the studies on pneumonia have reported a low prevalence of antibodies to *C. pneumoniae*. The results of our studies and of others (Falck 1997a), indicate that *C. pneumoniae* infection in children is most often confined to the upper respiratory tract, which could explain why studies of children with pneumonia have in general detected a low prevalence of the bacterium.

In Paper I, two different sites were used for specimen collection and the detection rates were dissimilar: throat swabs were superior to nasopharyngeal swabs (24% vs. 7%). Since the nasopharyngeal route was more often used on younger individuals some infections by *C. pneumoniae* may have been missed. Specimen collection from *C. pneumoniae* from throat necessitates a vigorous scrubbing that nurses were loath to do on small children and preferred to take nasopharyngeal specimens. The prevalence of *C. pneumoniae* in children younger than five years with respiratory tract infections may thus have been higher than the 12% detected by PCR in Paper I. The difference in yield from the two collections sites have been verified in adults when used on the same individuals (Gnarpe 1997b).
The prevalence of *C. pneumoniae* detected by PCR differs between Papers I, II and III. In Paper I two different sampling sites were used, which could explain some of the difference. The detection rate from throat swabs in Paper I was nearly equal to the finding in Paper II. Another explanation may be that the study in Paper I was conducted during 20 months from October 1994 and the study in Paper II was done during few months in 1996. The endemic prevalence of *C. pneumoniae* in the whole population may change over time and this may influence the results of prevalence studies done on different occasions (Karvonen 1993). There is evidence of two waves of epidemics during the time period of 1990 to 1996 (Gnarpe 1999). The low detection rate by PCR in Paper III completed in 1998 may be explained in the same way. A seasonal difference between spring and autumn is reported in Paper II.

The populations described in Papers I and II should not be compared for determination of the prevalence of *C. pneumoniae* in sick and healthy children without recognising important differences: the different clinical settings, the sample collection site for PCR specimen and study time period.

The seroprevalence in our studies was higher than reported in most other studies, however, a higher prevalence has been described for healthy children in Sudan (Herrmann 1994) and in children with pneumonia during a treatment trial in USA (Block 1995). The present study was conducted in another geographic region and the endemic prevalence of *C. pneumoniae* in this specific area may have been different, thus explaining a discrepancy in prevalence. Furthermore, there was an ongoing epidemic during a part of the study period.

When using IHC on biopsies from the upper respiratory tract of children, *C. pneumoniae* was detected in almost all adenoids. The children investigated all had chronic diseases confined to the upper respiratory tract and were in need of adenoidectomy. It could not be determined if *C. pneumoniae* had an etiologic role in these diseases or if this detection rate can be found in other populations. The frequent finding of *C. pneumoniae* in the adenoids may be a result of specific properties of the lymphoid tissue which acts as a filter. Adenoidal lymphocytes produce less Th1 cytokines as compared to peripheral blood lymphocytes (Bernstein 1998). If the high detection rate of *C. pneumoniae* by IHC on adenoid tissue holds true for other children without chronic problems, the upper respiratory tract of pre-school children may be a reservoir for *C. pneumoniae*. This has been suggested for other respiratory tract pathogens (Trottier 1989, Forsgren 1996). However, Schmidt et al. found that persistence of *C. pneumoniae* was uncommon when tested with PCR on repetitive throat swab specimens (Schmidt 2002).

In the present study no differences between boys or girls could be detected regarding evidence of *C. pneumoniae* infection. This was true for all diagnostic methods used. Several authors have described a difference in
the prevalence of antibodies to *C. pneumoniae* between sexes in adults (Grayston 1992).

The present study found that *C. pneumoniae* was more commonly found in mothers to infected children than in fathers. We found that *C. pneumoniae* was detected more often in children with several siblings; the same finding is reported in an earlier serological study (Paltiel 1995). Contrary to an earlier held belief (Grayston 1994), in-house transmission is therefore likely. It seems that *C. pneumoniae*, like *C. trachomatis* is relatively inefficiently transmitted and that close proximity is important for the transmission. The high prevalence of *C. pneumoniae* found in several day-care centres indicate that the organism may be easily transmitted between individuals living close together; the same has been found for other populations (Kleemola 1988, Pether 1989). The high prevalence of *C. pneumoniae* in children attending day-care has recently been verified (Schmidt 2002).

*Chlamydia pneumoniae* was more frequently found in children with furred pets at home. There is no explanation for this finding. No natural animal vector is suggested for *C. pneumoniae*.

In summary, *C. pneumoniae* is a common finding in small children. Studies based solely on MIF will underestimate the true prevalence in small children since these children usually do not develop antibodies detectable with standard serologic tests.

**Clinical implications**

**Respiratory tract infections**

Forty-six percent of the children infected with *C. pneumoniae* in Paper I were diagnosed as having nasopharyngitis. *Chlamydia pneumoniae* was detected more often in children with upper respiratory tract infection than in children with infections involving the lower respiratory tract (19% vs. 11%). Children infected by this agent most often had a relatively mild disease as indicated by the low prevalence of pulmonary signs of infection and the fact that they were most often treated as outpatients. However, these patients may have been ill for a long period of time before attending the hospital. *Chlamydia pneumoniae* was detected by IHC in almost every child with long-standing upper respiratory tract pathology undergoing adenoidectomy.

One obvious limitation of both Paper I and III is the lack of healthy controls. Ethical and financial reasons prevented us from including control groups in these studies.

In a study by Falck *et al.* healthy children and children with respiratory tract infections were examined with PCR for *C. pneumoniae* during the same period of time (Falck 1997a). In this study it was found that PCR was
positive in 45% of children with respiratory tract infections and in 6% of healthy children. The authors concluded that *C. pneumoniae* is a common finding in children with respiratory tract infections and that most infections are localised to the upper respiratory tract. The same method of specimen handling and PCR technique that was used in that study was used in the present investigations.

In the study of day-care children reported in Paper II, no consistent clinical finding could distinguish children infected with *C. pneumoniae* from children with negative PCR tests. Thirty-three percent of all children participating in the study reported signs of respiratory tract infections at the time of investigation. It is logical to assume that other respiratory tract pathogens may have flourished at that time. At follow-up four years later, children with a positive PCR test did not report any significant illnesses that differed from the PCR negative children during the time period that had elapsed from examination at the day-care centre. On the contrary, children earlier infected with *C. pneumoniae* reported a lower incidence of otitis media (35% vs. 48%) and this may contradict the association between *C. pneumoniae* and otitis media found by others (Ogawa 1990, Storgaard 1997, Block 1997).

The result of the present study indicates that infections with *C. pneumoniae* may be difficult to distinguish clinically from other trivial respiratory tract infections that encounter small children. Prolonged duration of relatively mild disease may be suggestive for symptomatic *C. pneumoniae* infection in children.

### Asymptomatic infections

The study in Paper II described children infected with *C. pneumoniae* without any clinical signs of infection. Sixteen percent of PCR positive children had no symptom of respiratory tract infection from the week before PCR analysis until the day of clinical examination and blood sampling, usually a time span of 2 months. In addition, 15% of children with detectable antibodies reported no sign of infection during the same time period. Consequently, asymptomatic infections may occur in child populations (Falck 1997a, Schmidt 2002).

*Chlamydia pneumoniae* may be detected in the upper respiratory tract in subjectively healthy adults (Gnarpe 1991, Hyman 1995). Adult individuals may seroconvert without having clinical disease during epidemics among people living in close proximity (Kleemola 1988, Berdal 1992). It is suggested that asymptomatic cases may transmit the organism to other individuals (Berdal 1997).

All children investigated in Paper I and III had some type of respiratory tract infection, which would exclude them from the study of asymptomatic infection.
Children may be infected with *C. pneumoniae* without having any symptoms. The rate of persistence in these subjects and the rate of transmission from asymptomatic individuals remain to be determined.

**Asthma and allergy**

Weak associations between *C. pneumoniae* and asthma were reported in Papers I and II. A pre-school-aged boy with asthma was followed for an extended period of time due to several episodes of exacerbations. This boy had high titres to *C. pneumoniae*, which increased during clinical deterioration. He was repetitively positive by PCR and he recovered after antibiotic treatment. Thus, *C. pneumoniae* infection may be associated with acute episodes of asthma in some individuals (Esposito 2000).

Despite the findings in this boy no relationship was found between *C. pneumoniae* and asthma when tested in a matched case-control study, including 285 cases and controls respectively (Paper V). This study was conducted on a large cohort. In the whole cohort a sub-population of girls with *C. pneumoniae* associated with asthma was found. These girls had certain specific findings in their humoral response related to *C. pneumoniae*: high specific IgG titres and the lack of specific IgA increased the risk for asthma. High titres are detected in asthmatics (v Hertzen 1999) but the IgA phenomenon is puzzling: anti-*C. pneumoniae*-IgA is associated with chronic infections (Saikku 1992) and has been found in association with asthma (Hahn 1996).

Although not statistically significant, we found antibodies to *C. pneumoniae* more commonly in non-sensitised than in sensitised asthmatics. von Hertzen et al. found that *C. pneumoniae* was especially associated with non-atopic asthma (v Hertzen 1999).

In Paper IV, PCR positive children were followed and monitored for any development of asthmatic disease during the four years that had elapsed. The results indicated a negative relationship; the odds ratio was 0.27 (0.03–2.35) for developing asthma if earlier PCR positive for *C. pneumoniae*. This suggested that children with heredity for asthma who were formerly negative in PCR tests were at highest risk to acquire asthma.

Most studies on associations of *C. pneumoniae* and asthma have been by serology (Hahn 1999). Associations have been found between asthma and a high incidence of *C. pneumoniae* infection (Emre 1994, Cunningham 1998, Esposito 2000), and with specific IgE antibodies suggested to influence atopic disease (Emre 1995, Ikezawa 2001). However, other studies contradict these findings (Larsen 1998, Mills 2000, Ferrari 2002).

The study in Paper V was done on four-year-old children. This is the age when the prevalence of children with detectable antibodies starts to increase, according to the results of this study. These children may still be too young...
for a study of an association between antibodies for *C. pneumoniae* and asthma.

The results in Papers IV and V display contradictory associations between *C. pneumoniae* and asthma. An earlier positive PCR test decreased the risk for the development of asthma while serologic responses to *C. pneumoniae* were related to a positive association, although the tested associations were statistically weak. If the findings are true, disparate results may be due to the different diagnostic methods used, detecting bacteria or specific antibodies.

Earlier infection with *C. pneumoniae* as detected by PCR seemed to reduce the risk for later atopy. Allergy was more common in the former PCR negative population, especially in children with atopic heredity. In Paper IV, the odds ratio was 0.12 (0.02–0.95) for acquiring allergic airway disease if the PCR test had been positive four years earlier. The mechanisms for this are unknown. Perhaps *C. pneumoniae* infection may protect some individuals from developing atopic disease or perhaps children with detectable *C. pneumoniae* infection have a specific immunologic constitution, including a lower risk for atopic disease. Bilenki *et al.* have reported the finding of inhibited eosinophilic inflammation after earlier infection with *C. trachomatis* and this may also hold true for *C. pneumoniae* (Bilenki 2000).

In Paper V, we found some indications supporting an inverse association between allergy and *C. pneumoniae* infection: antibodies to *C. pneumoniae* were less frequently found in sensitised children.

No strong evidence for an association between *C. pneumoniae* and asthma could be found in the overall study. However, findings suggest that *C. pneumoniae* may be of importance in some susceptible individuals, but the characteristics of these persons are as yet unknown. It was found that evidence of former *C. pneumoniae* infection as detected by PCR might reduce the risk for allergic disease.

**Treatment**

This study was not conducted to investigate the treatment of *C. pneumoniae* infection but the subject merits a short discussion. The use of antibiotics was documented in the studies in Paper II–IV. No significant information was given in Paper II and IV but some results were obtained in Paper III. In this study the five PCR positive children had been treated with antibiotics more frequently than other children. They had been given a median of 8 courses of antibiotics as compared with 4 courses in the PCR negative group (p<0.05, Kruskal-Wallis one-way analysis of variance by ranks for two groups). All children treated with macrolide antibiotics during the month prior adenoidealctomy were negative in PCR test, but not in the IHC.

Penicillin inhibits infectivity of *C. pneumoniae* but not viability (Kuo 1988). Macrolide antibiotics are recommended therapy for *C. pneumoniae*
infection in children (Malinverni 1995, Block 1995). However, after successful treatment as confirmed by culture, evidence of *C. pneumoniae* DNA may still be detected in some cases; this could represent the persistence of viable organisms in an unculturable state or could be a reflection of detection of persistent DNA remnants from successfully killed organisms (Malinverne 1995).

No conclusions on treatment can be drawn from the present study. There is great concern about increasing antibiotic resistance in several types of medically significant bacteria. Until further knowledge is learned about *C. pneumoniae*, it is advisable to treat only significant clinical diseases and to refrain from treatment when bacteria are detected in relatively healthy individuals.

**Other diseases**

*Chlamydia trachomatis* initially gives rise to a mild disease but persistent or repetitive infections over a longer period of time give rise to chronic sequelae such as blindness or infertility. If these characteristics for *C. trachomatis* also hold true for *C. pneumoniae*, most initial infections would be mild or asymptomatic. These infections may occur during childhood. At the present time, the most extensive research concerning *C. pneumoniae* concentrates on the organism’s role in the development in arteriosclerosis. The bacterium’s affinity for endothelial cells is well established. *Chlamydia pneumoniae* has been detected in cardiovascular tissue in young children (Normann 1999). As a result of *C. pneumoniae* infection, it may be speculated that arteriosclerosis associated with *C. pneumoniae* infection may constitute a parallel to trachoma and salpingitis with *C. trachomatis* infection.
CONCLUSIONS

- *Chlamydia pneumoniae* is commonly found in populations of young children

- *Chlamydia pneumoniae* transmits easily among people living close together

- Infections with *C. pneumoniae* in small children are often mild or asymptomatic

- Infections with *C. pneumoniae* may be of long duration

- No subsequent illness after *C. pneumoniae* infection in small children is identified

- Small children are not prone to develop detectable antibodies when infected with *C. pneumoniae*

- Further studies are needed to elucidate if early *C. pneumoniae* infection has any implication on future health
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)