EXPERIMENTAL CHLAMYDIA PNEUMONIAE INFECTION MODEL: EFFECTS OF REPEATED INOCULATIONS AND TREATMENT

LIISA TÖRMÄKANGAS

Faculty of Medicine,
Department of Medical Microbiology,
University of Oulu;
National Public Health Institute,
Department of Viral Diseases
and Immunology

OULU 2005
LIISA TÖRMÄKANGAS

EXPERIMENTAL CHLAMYDIA PNEUMONIAE INFECTION MODEL: EFFECTS OF REPEATED INOCULATIONS AND TREATMENT

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium of Kastelli Research Center (Aapistie 1), on January 21st, 2006, at 10 a.m.

OULUN YLIOPISTO, OULU 2005
Törmäkangas, Liisa, Experimental Chlamydia pneumoniae infection model: effects of repeated inoculations and treatment
Faculty of Medicine, Department of Medical Microbiology, University of Oulu, P.O.Box 5000, FIN-90014 University of Oulu, Finland; National Public Health Institute, Department of Viral Diseases and Immunology, P.O. Box 310, FI-90101 Oulu, Finland
2005
Oulu, Finland

Abstract

Chlamydia pneumoniae is a common human pathogen worldwide, which causes both upper and lower respiratory tract infections. In addition, C. pneumoniae infections have been associated with atherosclerosis and other chronic diseases, and successful treatment and eradication of the organism from tissues would therefore be desirable. The purpose of this study was to assess the effects of C. pneumoniae inoculations on the development of chronic infection and atherosclerotic changes in normocholesterolemic, wild-type mice. We also aimed to elucidate the effects of antibiotic and other treatments on the eradication of chlamydia and on the reduction of the pathologic sequelae induced by these infections.

Female C57BL/6J mice were fed either normal chow when assessing the effects of acute infection, or a diet supplemented with 0.2% cholesterol when evaluating the atherosclerotic changes. Primary or repeated inoculations with C. pneumoniae isolate K7 were given to the mice intranasally, and the effects of treatments with telithromycin, levofloxacin and erythromycin antimicrobial agents and with the phenolic compounds quercetin, luteolin and octyl gallate were evaluated. The following methods were used to measure infection and treatment effects and the presence of chlamydia in tissue: chlamydia culture, PCR and RT-PCR methods, histology of lung, heart and aortic tissue, serologic methods and measurements of aortic contractility responses.

Repeated C. pneumoniae inoculations induced persistent chlamydial DNA and inflammation in lung tissue and development of mouse Hsp60 autoantibodies. Infection was shown to influence aortic endothelial function, and repeated inoculations significantly increased subendothelial lipid accumulation in the aortic sinus area. A flavonoid, luteolin, was shown to effectively decrease the chlamydial load and inflammatory reactions in lung tissue. All antimicrobial agents eradicated the presence of viable chlamydia effectively; however, PCR positivity persisted in lung tissue despite the treatments. Only immediate treatment after each inoculation was able to decrease aortic sinus lipid accumulation.

In conclusion, these data support the role of C. pneumoniae in promoting atherosclerotic development via autoimmune responses and also via direct effects on aortic tissue. Conventional antimicrobial treatments may not effectively eradicate persistent infection, and further studies are warranted to seek for alternative treatment options.

Keywords: antibacterial agents, aorta, atherosclerosis, C57BL mice, Chlamydia pneumoniae, chronic disease, flavonoids, persistent infection, pneumonia, vascular endothelium
Acknowledgements

This work was carried out at the National Public Health Institute (KTL) Department of Viral Diseases and Immunology in Oulu and the Department of Medical Microbiology at the University of Oulu during the years 2000-2005. I thank the former Head of the Institute, Professor Jussi Huttunen, and the present Director General, Professor Pekka Puska, for providing good research facilities at KTL.

The studies were supervised by Professor Maija Leinonen and Professor Pekka Saikku, and I wish to express my sincere gratitude to them for all the knowledge and guidance they have provided me during these years. They have given me an opportunity to work on this fascinating area of microbiology, and I am deeply indebted to them for the encouragement, time and inspiring moments they provided in abundance.

I am grateful to the referees of the thesis, Docent Kimmo Mattila from the Hospital District of Helsinki and Uusimaa and Professor Ignatius W. Fong from the St. Michael’s Hospital, University of Toronto, Canada, for their thorough review of the manuscript. Their expertise and valuable and constructive comments greatly helped me to improve this work. I also want to thank Sirkka-Liisa Leinonen for her efficient and careful revision of the English language of the original articles and the thesis.

I thank Professor Tapani Hovi, Head of the Department of Viral Diseases and Immunology at KTL, for his interest and valuable comments on the original articles. I am also grateful to Liisa Pyhälä, Head of the Laboratory Animal Unit at KTL, for her expertise, guidance and advice in animal studies. She also provided excellent facilities for the animal studies together with the experienced staff of the unit.

I want to acknowledge the co-authors of the original articles who all contributed significantly to these studies: Denise Bem David and Andre Brysiker, who worked at Aventis Pharma, France, at the time of the studies, our animal pathologist Elise Saario, Hannu Alakärppä, the other PhD students in our group Leena Erkkilä, Taina Korhonen and Terttu Tiirola, Aini Bloigu who is especially acknowledged for her help and advice in statistical analyses, Professor Pia Vuorela from Åbo Akademi, Professor Heikki Vuorela from the Faculty of Pharmacy, University of Helsinki, and Juha Ketonen and Professor Ilari Paakkari from the Institute of Biomedicine, University of Helsinki. I also thank Dr. Juri Yamakita from Daiichi, Japan, for his co-operation and advice with the Levofoxacin experiments.
Several people have helped me with the laboratory analyses of these studies. I appreciate their skilful work and experience at the lab, and they deserve my unreserved acknowledgements. Especially, I want to thank Maija Holtinkoski, who taught me all about culturing chlamydia at the beginning of my studies. Mika Paldanius, Anne Jaakkola and Annikki Törmälehto also advised and assisted me with chlamydia culture and serology; Leena Kuisma and Anu Ojala did a great job analysing the numerous DNA samples, Hannele Huumonen helped a lot with the chlamydial RNA analyses, and Terttu Korpela did most of the histology in these studies. Minttu Ahjos and Sirke Haakalindgren did invaluable work in Helsinki at the Laboratory Animal Unit by giving the majority of treatments to the mice and taking care of many of the practical aspects of the animal experiments.

Docent Sylvi Silvennoinen-Kassinen and Docent Virpi Glumoff from the Department of Medical Microbiology, University of Oulu, who belong to the follow-up group of this thesis, are acknowledged for their interest and advice in this process.

Many thanks also go to the staff at KTL in Oulu for always being nice and supportive and helping in many ways. Raili Voittonen is acknowledged for her help in obtaining scientific articles from various sources. Tiina Sävykoski, Annika Saukkoriipi, Leena Erkkilä and Taina Korhonen have been both colleagues and good friends. Thank you all for the advice at the lab as well as for the enormous amounts of laughter you have provided during these years.

My dearest friends and beloved ones, Riikka and Juha, Sari, Tiina, Marjo, and Erkki, you have been there to share many of the best and the most enjoyable moments with me both above and below the surface during this project. I am also most fortunate to have my caring and supportive parents and brothers with their families around me; you have all made this possible in such many ways. Thank you.

The studies were financially supported by the Academy of Finland, Aventis Pharma, Daiichi, the Finnish Konkordia Fund, the Finnish Society for Study of Infectious Diseases, the Graduate School of Circumpolar Wellbeing, Health and Adaptation, Paulig Group/Control-ox Ltd., Retro Life Assurance Company Ltd., the Sigrid Jusélius Foundation, and Tekes – the National Technology Agency of Finland.

Oulu, November 2005

Liisa Törmäkangas
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</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>enzymeimmunoassay</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HE</td>
<td>haematoxylin-eosin</td>
</tr>
<tr>
<td>Hsp60</td>
<td>60 kDa heat shock protein</td>
</tr>
<tr>
<td>IFU</td>
<td>inclusion-forming unit</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LGV</td>
<td>lymphogranuloma venereum</td>
</tr>
<tr>
<td>L-NAME</td>
<td>Nω-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MBC</td>
<td>minimal bactericidal concentration</td>
</tr>
<tr>
<td>MC</td>
<td>methacholine</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>MIF</td>
<td>microimmunofluorescence</td>
</tr>
<tr>
<td>MOMP</td>
<td>major outer membrane protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>Omp</td>
<td>outer membrane protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Pmp</td>
<td>polymorphic membrane protein</td>
</tr>
<tr>
<td>RB</td>
<td>reticulate body</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cells</td>
</tr>
<tr>
<td>SPG</td>
<td>sucrose-phosphate-glutamic acid</td>
</tr>
<tr>
<td>TRF</td>
<td>time-resolved fluorescence</td>
</tr>
<tr>
<td>UNG</td>
<td>uracil-DNA-glycosylase</td>
</tr>
</tbody>
</table>
List of original publications

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


V Törmäkangas L, Ketonen J, Leinonen M, Saikku P & Paakkari I. Increased prostanoid dependency of arterial relaxation in *Chlamydia pneumoniae*-infected mice. Submitted for publication.

Some previously unpublished data are also presented. The original articles are reprinted with permission from British Society for Antimicrobial Chemotherapy, Oxford University Press (I); Copyright © American Society for Microbiology (II, III), and Elsevier (IV).
Contents
Abstract
Acknowledgements
Abbreviations
List of original publications
Contents
1 Introduction ................................................................................................................... 13
2 Review of the literature ................................................................................................. 15
  2.1 Chlamydiaceae ........................................................................................................ 15
    2.1.1 Strucutre ............................................................................................................ 17
    2.1.2 Developmental cycle ....................................................................................... 18
    2.1.3 Clinical manifestations of acute infections ...................................................... 20
      2.1.3.1 C. pneumoniae ......................................................................................... 20
      2.1.3.2 C. trachomatis ......................................................................................... 21
      2.1.3.3 Chlamydiae with natural animal hosts ...................................................... 22
    2.1.4 Chronic infections ......................................................................................... 22
  2.2 Pathogenesis of C. pneumoniae infections ............................................................. 24
  2.3 Animal models of C. pneumoniae infection ........................................................... 25
  2.4 Atherosclerosis ...................................................................................................... 27
    2.4.1 Atherosclerosis animal models ................................................................. 28
    2.4.2 C. pneumoniae and atherosclerosis ............................................................ 29
    2.4.3 Other microbes associated with atherosclerosis and cardiovascular diseases ......................................................................................... 32
  2.5 Antibiotic treatment of chlamydial infections ....................................................... 32
    2.5.1 Animal models for C. pneumoniae treatment .............................................. 35
    2.5.2 Human studies with antibiotic treatment ..................................................... 36
  2.6 Antichlamydial potential of other compounds ..................................................... 39
    2.6.1 Flavonoids ..................................................................................................... 39
    2.6.2 Cathelicidin peptides ................................................................................... 40
    2.6.3 Statins ........................................................................................................... 40
  3 Aims of the study ....................................................................................................... 42
  4 Materials and methods .............................................................................................. 43
1 Introduction

Chlamydiae are obligate intracellular pathogens detected widely in humans, but also in other mammals, reptiles and birds. Besides acute infections, these bacteria are known to cause chronic disease sequelae due to either repeated infections or persistent presence of the organism in tissue. *Chlamydia pneumoniae* is mainly a human respiratory tract pathogen, and serological data indicate that 40 to 70% of the adult population worldwide have detectable antibodies against it. Most infections are mild and undiagnosed, with rhinitis, sore throat and cough, but pneumonia, bronchitis, pharyngitis, sinusitis and otitis are also caused by *C. pneumoniae*. (Grayston et al. 1990) Acute respiratory tract infections are commonly treated with prolonged courses of macrolide, tetracycline, or fluoroquinolone antibiotics, but treatment failures and persistent infections are occasionally reported (Hammerschlag et al. 1992, Falck et al. 1996, Miyashita et al. 2003b).

*C. pneumoniae* is also able to spread systematically from the respiratory tract to various other tissues, most probably via circulating blood mononuclear cells (Moazed et al. 1998, Bodetti & Timms 2000), and it is able to infect several different cell types (Godzik et al. 1995, Gaydos et al. 1996, Kaukoranta-Tolvanen et al. 1996b). These findings together with a wide variety of serologic and other data showing, for instance, the presence of viable organisms in diseased aortic tissues support the role of *C. pneumoniae* in the development of atherosclerosis (Leinonen & Saikku 2002, Kalayoglu et al. 2002), as first proposed almost 20 years ago (Saikku et al. 1988).

The persistent, aberrant form of chlamydiae can be induced by antibiotics and various other methods *in vitro* (Hogan et al. 2004), and similar persistence may also be present *in vivo* (Nanagara et al. 1995, Gerard et al. 1998, Hogan et al. 2004). There are several studies to indicate that persistent infections caused by chlamydiae may not be responsive to antimicrobial agents (Oriel & Ridgway 1982, Kutlin et al. 1999, Gieffers et al. 2001, Baltch et al. 2004, Reveneau et al. 2005), and many of the large-scale antibiotic treatment trials in humans targeting *C. pneumoniae* have failed to protect the patients from subsequent cardiovascular events (Andraws et al. 2005). Further studies elucidating the effect of combined or alternative treatments against persistent infections as well as vaccine development are therefore needed.
Animal models have shown *C. pneumoniae* to be able to induce or accelerate atherosclerotic development in rabbits (Laitinen *et al.* 1997, Fong *et al.* 1999b) and hypercholesterolemic mice (Hu *et al.* 1999, Moazed *et al.* 1999). Yet, an atherosclerotic diet and genetic manipulations may induce additional confounding effects in mice, as shown earlier (Liao *et al.* 1993, Roselaar & Daugherty 1998, Laskowitz *et al.* 2000, de Bont *et al.* 2000). In this study, wild-type mice were used to study the effects of *C. pneumoniae* infection on the induction and early events of atherosclerotic development and to test the effects of different treatments on these changes. It was shown previously in rabbits (Muhlestein *et al.* 1998, Fong *et al.* 1999a), but not in apolipoprotein E (ApoE) knock-out mice (Rothstein *et al.* 2001, Blessing *et al.* 2005), that antimicrobial treatments are able to reduce the atherosclerotic development induced by *C. pneumoniae*. 
2 Review of the literature

2.1 Chlamydiaceae

Originally thought to be viruses due to their obligate intracellular demands and ability to pass filters generally retaining bacteria, chlamydiae began to be recognised as a specific group of bacteria after studies of the chlamydial cell wall structure by electron microscopy, detection of the presence of both RNA and DNA, and demonstration of the division of particles by binary fission (Moulder 1964). Isolation of this agent as a causative agent of trachoma was first accomplished in 1957 in China, using chick embryo yolk sacs (T’ang et al. 1957). After differentiation of Chlamydiae from the Rickettsiae group, only two species were recognised within the family Chlamydiaceae and the genus Chlamydia in the course of more than 20 years (Page 1968); C. trachomatis strains that were capable of accumulating glycogen in the inclusion and were sensitive to sulfadiazine and C. psittaci strains that did not accumulate glycogen and were mainly resistant to sulfadiazine.

Since 1957, several new chlamydial strains have been isolated - one of them the TW-183 strain isolated from an eye of a child in Taiwan in 1965 - which were found to be a serologically unique group among the known C. psittaci isolates. These showed differential, milder pathogenic properties when grown in cell cultures or inoculated into mice or chicken embryos. This group of organisms was eventually called TWAR after the isolates TW-183 and AR-39 (from a throat swab of a patient with pharyngitis in 1983). The strains were associated with epidemics of respiratory tract infections and pneumonia with no known avian source, which finding was first published based on a Finnish material (Saikku et al. 1985). Earlier, Darougar and co-workers had reported that approximately one fifth of the adult population (blood donors) in London had antibodies against an atypical Iranian chlamydial isolate designated as IOL-207 (Darougar et al. 1980). The association with pneumonia epidemics and the presence of antibodies against these unusual strains in considerable numbers of adult populations led to the proposal of TWAR organisms being primarily a human pathogen (Darougar et al. 1980, Saikku et al. 1985, Grayston et al. 1986, Kuo et al. 1986). Finally, a new species including antigenically uniform isolates, C. pneumoniae, was proposed in 1989 (Grayston et al.
16

1989a), and it was later generally accepted within the genus. Still, a considerable number of isolates within the C. psittaci species made up a divergent group of chlamydiae with a wide spectrum of hosts, including birds and non-human mammals. A fourth species of the genus Chlamydia, C. pecorum, was proposed in 1992, based on genetic and serological analyses, to belong to a separate group of organisms isolated from cattle and sheep (Fukushi & Hirai 1992).

Comparative phylogenetic analyses of several isolates within the four species of Chlamydia using 16S ribonucleic RNA (rRNA) sequences (Pudjiatmoko et al. 1997) and DNA sequences for the chlamydia major outer membrane protein (MOMP) gene (ompA) (Kaltenboeck et al. 1993) suggested two separate clusters in the genus: one cluster consisting of the C. trachomatis strains and the other of the C. pneumoniae, C. psittaci and C. pecorum strains. Based on their results, and in accordance with the report of Kaltenboeck et al., Pudjiatmoko and co-workers proposed the C. trachomatis cluster and the C. psittaci strains both to be further divided. The C. trachomatis human strains within the trachoma and lymphogranuloma venereum (LGV) biovars were shown to be more similar to each other, whereas the strains in the mouse and swine (originally considered as C. psittaci) biovars consisted of two additional genetic groups. Further, the C. psittaci strains were proposed to belong to three separate genetic groups as well, the avian and abortus strains, the guinea pig strains and the feline strains. Similar subgroup divisions of C. psittaci and C. trachomatis have also been proposed in other studies based on restriction fragment length polymorphism of the 16S-23S rRNA spacer region (Meijer et al. 1997) and on whole genomic amplified fragment length polymorphism (Meijer et al. 1999).

Later, in 1999, a completely new taxonomy for chlamydiae was proposed (Everett et al. 1999). Within the family Chlamydiaceae, a new genus Chlamydophila was included, which contains the former C. pneumoniae, C. pecorum and C. psittaci species (now named Chlamydophila) and also the three new species separated from C. psittaci strains: Chlamydophila abortus, Chlamydophila felis and Chlamydophila caviae. Another genus, the former Chlamydia, was proposed to include C. trachomatis (the trachoma and LGV biovars), Chlamydia muridarum (the murine biovar) and Chlamydia suis (the swine biovar). In addition, two new families, Simcaniaceae and Parachlamydiaceae, were included in the order Chlamydiales to place the newly identified chlamydia-like organisms (Kahane et al. 1995, Amann et al. 1997). Although the reclassification provided the necessary revision of the chlamydia taxonomy, especially in view of the divergence within the C. psittaci strains, the radical proposal was not generally accepted by chlamydiologists (Schachter et al. 2001). The main arguments were levelled against the division of the species into two separate genera, which is not necessarily supported by the genomic differences between the species. Especially, the almost sole use of sequence differences in the 16S and 23S rRNA genes as a basis of the reclassification and the criteria of less than 95% identity in the sequences subdividing the species, which is used in the determination of the phylogenies of some free-living bacteria, were criticised. Isolated intracellular bacteria with no obvious horizontal gene transfer and with increased rates of mutations should not be classified as free-living bacteria. (Stephens 2002). In agreement with the arguments opposing the genus division, the old classification including only one genus, Chlamydia, has been used in the present studies.
2.1.1 Structure

Chlamydiae are unique pathogenic intracellular organisms with a specific developmental cycle. The extracellular, infective form of the bacteria is termed elementary body (EB) and the intracellular, dividing form reticulate body (RB). The envelope of the gram-negative chlamydial particle consists of two layers, the inner lipid layer and the surface layer containing lipopolysaccharide (LPS). Unlike in most gram-negative bacteria, peptidoglycan, which is responsible for the osmotic integrity of bacteria, is not present in the chlamydial cell wall (Moulder 1993). Early studies attempting to detect N-acetylmuramic acid, one of the peptidoglycan components, in chlamydiae EBs yielded discrepant results, and only trace amounts of muramic acid were detected in some of these studies, as reviewed in (Fox et al. 1990). Yet, it has been known for a long time that the growth of chlamydiae is restricted by penicillin (Matsumoto & Manire 1970) and D-cycloserine (Moulder et al. 1963) treatments, and recently, analyses of chlamydial genomes have revealed chlamydiae to possess nearly the complete set of genes required for the synthesis of peptidoglycan (Stephens et al. 1998). Recent studies with chlamydial proteins functioning as enzymes in the peptidoglycan synthesis expressed in Escherichia coli have confirmed the suggestions that peptidoglycan may be present in chlamydial RBs and is needed for RB division (McCoy et al. 2003, Hesse et al. 2003).

The main components of the chlamydial outer membrane complex are the 39.5 kDa MOMP (Caldwell et al. 1981), the 60 and 12.5 kDa cystein-rich outer membrane proteins (Omp) Omp2 and Omp3 (also known as OmcB/EnvB and OmcA/EnvA, respectively) (Newhall et al. 1982, Hatch et al. 1984), chlamydial LPS (Nurminen et al. 1983, Caldwell & Hitchcock 1984), and a variable number of more recently discovered polymorphic membrane proteins (Pmps). Pmps were originally discovered and thought to be present only in the C. pneumoniae and C. psittaci strains (Campbell et al. 1990a, Perez Melgosa et al. 1993), but the analysis of chlamydial genomes has identified nine Pmp genes in C. trachomatis and altogether 21 genes in C. pneumoniae (Stephens et al. 1998, Kalman et al. 1999). All of the 21 genes found in the C. pneumoniae genome have been shown to be transcribed, but apparently not all of them are expressed (Grimwood et al. 2001, Vandahl et al. 2001). The exact function of the Pmps is not known, although several studies indicate that some Pmps are potentially surface-exposed and immunogenic (Longbottom et al. 1998, Knudsen et al. 1999, Tanzer et al. 2001). In addition, the possible role of Pmps as autotransporters has been proposed (Henderson & Lam 2001, Vandahl et al. 2002b).

Chlamydial LPS is the genus-specific group antigen, which is known to be surface-exposed and is present in both EBs and RBs (Brade et al. 1997). The rough phenotype detected is similar to the Re-LPS in the mutant strains of E. coli and Salmonella spp. (Nurminen et al. 1985), but chlamydial LPS is a far less potent endotoxin and inducer of an acute immune response (Ingalls et al. 1995). Unlike LPS, the cystein-rich proteins Omp2 and Omp3 are both synthesised late in the developmental cycle and are present only in EBs (Hatch et al. 1984, Hatch 1996). In C. trachomatis and in C. psittaci, and probably in C. pneumoniae as well, Omp3/EnvA is suggested to be anchored by its lipid moiety to the outer membrane on the periplasmic side, and Omp2/EnvB is presumably completely periplasmic (Everett et al. 1994, Everett & Hatch 1995, Hatch 1996, Mygind
et al. 1998). The disulfide bonds of these cystein-rich proteins and MOMP are considered to maintain the structural integrity of EBs lacking the peptidoglycan. MOMP, being the most abundant protein in the outer membrane of chlamydiae, is also considered to form pore-like structures, depending on the disulfide bonding, through the outer membrane (Bavoil et al. 1984, McCafferty et al. 1995, Wyllie et al. 1998). In C. trachomatis, MOMP is surface-exposed and the major immunodominant antigen containing four variable domains, which are the basis for serotype differentiation (Newhall et al. 1982, Stephens et al. 1987, Bachr et al. 1988). In contrast, the MOMP of C. pneumoniae, unlike that of also C. psittaci (Zhang et al. 1989), is shown to be highly conserved between the different isolates (Gaydos et al. 1992a). Recent studies have shown that the MOMP of C. pneumoniae is indeed surface-exposed (Peterson et al. 1996, Wolf et al. 2001), though it has been thought to be only weakly or not at all immunogenic (Campbell et al. 1990b, Peterson et al. 1996). More recently, Wolf and co-workers showed that neutralising antibodies are actually produced against surface-exposed C. pneumoniae MOMP epitopes, but these epitopes are conformational and easily destroyed by detergents when the antigens are processed for immunoblotting or other analyses (Wolf et al. 2001). This was also suggested by Puolakkainen and colleagues in an earlier study (Puolakkainen et al. 1995).

Increasing evidence suggests that the type III secretion (TTS) system known in other gram-negative pathogenic bacteria, such as Yersinia and Salmonella, would also be functional in chlamydiae. Hsia and co-workers first identified chlamydial genes homologous to the system (Hsia et al. 1997), and chlamydial genome analyses have shown the presence of a complete set of genes orthologous to the TTS genes in other bacteria (Stephens et al. 1998, Kalman et al. 1999). Proteomic analyses of C. pneumoniae demonstrated that all members of TTS are present in EB particles (Vandahl et al. 2001), whereas only one of them, YscC, was found in the outer membrane complex proteome (Vandahl et al. 2002a). This suggests it to be surface-exposed. Much earlier, Matsumoto and colleagues detected spike-like projections on the surface of C. psittaci by electron microscopy (Matsumoto 1982), and after the recognition of the first TTS genes in chlamydia, Bavoil and Hsia suggested these projections to represent the TTS system (Bavoil & Hsia 1998).

In addition to the structures already mentioned, other, as yet unspecified proteins have been proposed to be surface-exposed in C. pneumoniae: two proteins with molecular weights of 54 kDa (Freidank et al. 1993, Wiedmann-Al-Ahmad et al. 1997) and 76 kDa (Perez Melgosa et al. 1994) have been shown to be C. pneumoniae-specific and to induce antibodies which neutralized C. pneumoniae infection in vitro. Further studies are needed to reveal the function and exact location of these proteins.

### 2.1.2 Developmental cycle

The biphasic developmental cycle of chlamydiae initiates with the binding of the infectious EB, 0.2 to 0.3 µm in size, to the host cell membrane. Morphologically, EBs are dense particles generally with either round or pear-shaped (some C. pneumonia isolates) outer membrane and narrow periplasmic space (Miyashita & Matsumoto 2004) (Fig. 1A).
The receptors and chlamydial ligands involved in the attachment of EBs to cell membranes remain undefined, but several chlamydial structural components, e.g. heparin sulphate-like proteoglycans, MOMP or glycosylated MOMP, Omp2, heat shock protein (Hsp) 70 and Pmps have been suggested to function as adhesins, as reviewed in (Hackstadt 1999). Recently, Puolakkainen and co-workers suggested the mannose 6-phosphate/insulin-like growth factor 2 receptor to serve as a receptor for *C. pneumoniae* entry (Puolakkainen *et al.* 2005). After attachment, the EB is rapidly internalised into the host cell cytoplasm, and apparently, several ways are used for entering the cell: phagocytosis (Byrne & Moulder 1978), endocytosis in clathrin-coated pits (Wyrick *et al.* 1989) and pinocytosis in non-clathrin-coated pits (Prain & Pearce 1989). Inside the host cell, the EB within a modified vacuole, called inclusion, avoids fusion with lysosomes and vacuole modification. Trafficking of the EB to the perinuclear region and the Golgi area is dependent on early chlamydial gene expression. (Wyrick 2000).

Around six hours after their entry, EBs start to differentiate into RBs, and replication presumably by binary fission begins (Fig. 1B-C). RBs consist of homogenous internal material and are larger than EBs, about 1 µm in diameter. To be able to divide, chlamydiae are dependent on host cell iron, amino acids and energy (Wyrick 2000); however, there are differences in the specific needs for nutrients and energy between chlamydia species and strains. For example, chlamydial genome sequences have revealed that *C. psittaci* guinea pig inclusion conjunctivitis strains have the most complete set of genes required for tryptophan biosynthesis, whereas these genes are absent in the *C. pneumoniae* genome (Stephens *et al.* 1998, Kalman *et al.* 1999, Read *et al.* 2003). In addition, recent studies and the genome sequence indicate that at least *C. trachomatis* LGV serovars can metabolise glucose and produce ATP of their own by oxidative phosphorylation (Iliffe-Lee & McClarty 1999), thus compromising the concept of chlamydiae as being “energy parasites”.

During the intracellular phase, chlamydiae secrete several proteins, which obviously have important functions for chlamydial growth and survival. The Inc proteins are detectable in the inclusion membrane, whereas some proteins are secreted into the cytoplasm of the host cell, including the protease-like activity factor (CPAF). The Inc proteins in the inclusion membrane may affect inclusion development, avoidance of lysosomal fusion, vacuole trafficking and nutrient acquisition. CPAF has been shown, for example, to modify major histocompatibility complex expression, as reviewed in (Christiansen *et al.* 2004). By 24 hours in the case of *C. trachomatis* and around 36 hours in the case of *C. pneumoniae*, RBs start to reorganise back to EBs (Fig. 1D) and are finally released from the cell at 48 to 72 hours after the initiation of the cycle. The exact timing of the development cycle depends on the chlamydial species and strains as well as on the growth conditions and the cell lines used.
2.1.3 Clinical manifestations of acute infections

2.1.3.1 C. pneumoniae

Serologic studies indicate that C. pneumoniae antibodies are rarely detected in children under five years old, and that their prevalence increases rapidly between 5 and 15 years of age (Grayston 1992, Grayston 1994). Yet, some more recent studies suggest the presence of C. pneumoniae even in young children despite the lack of antibody response in the microimmunofluorescence (MIF) test (Normann et al. 1998, Kutlin et al. 1998). Around 40% to 70% of the adult population, as reported in several countries, have detectable levels C. pneumoniae antibodies (Grayston et al. 1990, Marton et al. 1992, Karvonen et al. 1994, Gencay et al. 1998, Ben-Yaakov et al. 2002, Shimizu et al. 2002), and even higher prevalences are seen in elderly populations (Grayston 1992, Koivisto et al. 1999). Thus, the majority of humans are infected with C. pneumoniae at some stage of their lives.

After transmission from person to person, generally via respiratory secretions and droplets, C. pneumoniae invades the epithelial cells of the respiratory tract and lung tissue. Transmission is relatively inefficient, and the incubation period may be several weeks, which slows down the spreading of outbreaks (Kleemola et al. 1988, Kuo et al. 1989).
C. pneumoniae causes a wide variety of respiratory infections, most of which are mild and self-restricted and therefore often undiagnosed, but also severe pneumonia, especially in the elderly people (Troy et al. 1997). Clinical findings in C. pneumoniae infections include dry cough, sore throat, hoarseness and rhinitis, which are frequently detected. Fever is not necessarily present in mild infections. (Grayston et al. 1990, Ekman et al. 1993). During non-epidemic seasons, reports have presented variable data on the association of C. pneumoniae infection with the different upper respiratory tract diseases, as reviewed earlier (Hahn et al. 1991, Falck et al. 1994) and about 9% of pharyngitis (Huovinen et al. 1989) cases involved C. pneumoniae, whereas involvement in sinusitis and otitis has been detected in some (Block et al. 1997, Storgaard et al. 1997, Falck et al. 1998) but not all of the reports and not in very young children (Jero et al. 1999, Storgaard et al. 2004). C. pneumoniae incidence rates between 3% and 16% of all community-acquired pneumonia cases in the adult population have been reported when no epidemics are present, as reviewed by (Hahn et al. 2002) and (Blasi et al. 2004). Similar rates have also been reported in children (Blasi et al. 2004, Michelow et al. 2004), although not in those younger than five years old (Liu et al. 2005). During C. pneumoniae epidemics, pneumonia, bronchitis and pharyngitis are commonly detected in patients (Kleemola et al. 1988, Fryden et al. 1989, Grayston et al. 1990, Ekman et al. 1993).

### 2.1.3.2 C. trachomatis

C. trachomatis strains have traditionally been divided into three variant groups, biovars, with differences in host species and target cells, disease sequelae and virulence. Two of these, the trachoma and LGV biovars, cause infections in humans. The Chlamydiae in the trachoma biovar are important pathogens as causative agents of trachoma and the most common sexually transmitted bacterial disease. The C. trachomatis serotypes from D to K infect columnar epithelial cells mainly in the urogenital tract, but infections of conjunctiva and respiratory tract epithelial cells are also characterised, especially in infants infected at birth. These serotypes are also known to cause occasional inclusion conjunctivitis in adults. The most common consequences of an acute chlamydial infection in women are cervicitis, salpingitis and urethritis. C. trachomatis is also one of the causative agents in pelvic inflammatory disease (PID), which commonly results in tubal factor infertility, ectopic pregnancies and chronic pelvic pain. 10% to 40% of C. trachomatis infections have been associated with the development of PID (Simms & Stephenson 2000). In men, male urethritis and epididymitis, a severe complication of acute urethritis, are the common outcomes of chlamydial infection, although most infections are asymptomatic both in men and in women. The disease sequelae seen in C. trachomatis infections are thoroughly reviewed in (Schachter 1999).

The C. trachomatis serotypes A, B, Ba and C cause conjunctivitis of the eye lids, which, after repeated infections, leads to fibrosis and scarring of the subtarsal conjunctiva, i.e. classic trachoma (Maibey et al. 2003). Repeated sequelae and distortion of the lids may cause the eyelashes to turn inward (trichiasis), creating physical damage to the cornea and, thereby, eventually leading to blindness. Trachoma is endemic in
tropical and subtropical countries and associated especially with poor hygienic conditions; it is considered the most common cause of preventable blindness in the world. (Schachter 1999).

The *C. trachomatis* strains in the LGV biovar (serotypes L1, L2 and L3) cause sexually transmitted systemic infections, where the organisms proliferate in lymph nodes and subepithelial tissues, a disease nowadays mainly seen in developing countries (Schachter 1999).

### 2.1.3.3 Chlamydiae with natural animal hosts

Apart from common human infections, chlamydia isolates closely related to *C. pneumoniae* have been found to cause mild infections in koalas (Glassick *et al.* 1996, Jackson *et al.* 1999), and these organisms have also been found in horses (Storey *et al.* 1993) and frogs (Berger *et al.* 1999). Whether these strains are able to cause infections in humans is not known, and further studies on this topic are warranted. Other chlamydiae are mainly animal pathogens, although *C. psittaci* avian strains have, since the beginning of recognition of chlamydiae, been known to be capable of causing epidemics of both upper respiratory tract infections and atypical pneumonia, psittacosis, in humans. Psittacosis is acquired via the respiratory route, and some strains are highly infective. The most common sources are parrots and parakeets, pigeons and poultry. (Schachter 1999). The disease is considered relatively rare in these days, although sporadic cases and occasional outbreaks are reported, especially among people working in close contact with birds (Telfer *et al.* 2005, Saito *et al.* 2005). Many of the early psittacosis outbreaks described may well have been caused by *C. pneumoniae*, as described in the Scandinavian ornithosis epidemics in the 1990’s (Grayston *et al.* 1989b, Fryden *et al.* 1989), since the complement fixation tests used to diagnose psittacosis detect both agents similarly.

Infections of humans with other than avian psittacosis species are rare. Still, the chlamydia agent causing spontaneous abortions in sheep (Entrican *et al.* 2001), goats (Rodolakis *et al.* 1998) and cattle (Kaltenboeck *et al.* 2005), called *C. psittaci* serovar 1 (*C. abortus* according to the new proposed taxonomy), has been associated with spontaneous abortions in women working in close contact with diseased animals (Pospischil *et al.* 2002, Meijer *et al.* 2004). In these cases, the abortion coincided with very rapidly worsening severe physical condition, including sepsicaemia or influenza-like illness. Similar rare cases have been reported occasionally, as reviewed earlier in (Hyde & Benirschke 1997). Some papers have also reported sporadic cases of human infections with feline chlamydial strains (Cotton & Partridge 1998, Marrie *et al.* 2003).

### 2.1.4 Chronic infections

In the case of all chlamydiae, persistent or repeated infections are associated with a wide variety of chronic diseases. As described previously, trachoma is a consequence of repeated *C. trachomatis* infections during several years or even decades, and sexually
transmitted chlamydial infections may lead to tubal scarring and infertility in women. The role of persistent versus repeated infections in the disease aetiologies are sometimes difficult to distinguish, and there are data indicating that infections caused by chlamydia may persist for long periods (Dean et al. 2000, Miyashita et al. 2003b), sometimes even despite appropriate treatment (Hammerschlag et al. 1992, Roblin et al. 1994, Falck et al. 1996, Miyashita et al. 2003b). Studies in which C. pneumoniae have been isolated from atherosclerotic coronary arteries or from patients with community-acquired pneumonia indicate that the strains were still susceptible to antimicrobial agents (Gieffers et al. 1998), although some increases were detected in the minimal inhibitory concentration (MIC) values (Roblin & Hammerschlag 1998b). Therefore, treatment failures are not considered to be due to the development of antimicrobial resistance. Further evidence of the persistence of chlamydial infections has been obtained from animal models: culture-negative, persistent chlamydia infection either in lung tissue or in the genital tract in mice, demonstrated as polymerase chain reaction (PCR) positivity after the primary inoculation, can be reactivated by immunosuppressive cortisone treatment (Yang et al. 1983, Malinverni et al. 1995a, Laitinen et al. 1996, Cotter et al. 1997).

In certain growth conditions, an aberrant but viable and persistent form of chlamydia can be induced in vitro by cytokines such as interferon-gamma (IFN-γ) (Beatty et al. 1993, Pantoja et al. 2001), penicillin (Matsumoto & Manire 1970), amino acid or iron starvation (Coles et al. 1993, Raulston 1997), phage infection (Hsia et al. 2000) and in a continuous infection model (Moulder et al. 1980, Kutlin et al. 2001). Besides β-lactam antibiotics, the first-line antibiotics erythromycin, doxycycline, ciprofloxacin and rifampin have also been shown to induce persistence when administered in subinhibitory concentrations (Gieffers et al. 2004) or after long-term treatment in the case of fluoroquinolones (Dreses-Werringloer et al. 2000). Another model for persistent and aberrant forms of chlamydia and restricted growth is infection in normal human monocytes, which leads to a non-productive infection with viable chlamydia in these cells (Koehler et al. 1997, Airenne et al. 1999). These forms resemble RBs but are larger in size after genomic replication and without actual division of the particle. The gene expression pattern is shown to be different from that of the actively growing chlamydia in both C. pneumoniae (Byrne et al. 2001, Mathews et al. 2001) and C. trachomatis (Gerard et al. 2001, Gerard et al. 2002) infections. In general, the genes needed for replication are functional or even up-regulated, whereas the genes for cytokinesis and energy metabolism are down-regulated. Up-regulation of the protein expression of Hsp60 and down-regulation of MOMP are also detected in vitro (Beatty et al. 1994b). Furthermore, the gene (groEL) coding C. pneumoniae Hsp60 protein has been shown to be up-regulated in murine alveolar macrophages (Haranaga et al. 2003) in some (Dean & Powers 2001, Molestina et al. 2002) but not all (Mathews et al. 2001, Belland et al. 2003) previous in vitro studies after IFN-γ-induced persistence of infection. IFN-γ is thought to induce persistence by induction of indoleamine 2,3-dioxygenase enzyme, which catalyses intracellular tryptophan. Depletion of this essential amino acid restricts chlamydial growth (Beatty et al. 1994a). Removal of the interfering factor, for example IFN-γ, from the culture generally normalizes the developmental cycle, and the formation of infectious EBs is restored (Beatty et al. 1993, Beatty et al. 1995). Interestingly, tobacco smoke was also shown to cause formation of this aberrant chlamydia in vitro (Wiedeman et al. 2004). It is not clear yet whether this form of experimental persistence
really occurs in vivo in clinical diseases, but there are studies to suggest the presence of viable, abnormal chlamydia in the synovial tissue of patients with Reiter’s syndrome (Nanagara et al. 1995).

In addition to the chronic sequelae at the site of infection, C. trachomatis is the primary urogenital pathogen involved in the development of reactive arthritis (Kousa et al. 1978, Villareal et al. 2002). It has been shown that chlamydiae are both viable and metabolically active in joints, and importantly, the gene expression pattern indicates a persistent but not an actively growing form of chlamydia at the inflammation site (Gerard et al. 1998, Gerard et al. 2002). The presence of C. pneumoniae DNA and messenger RNA (mRNA) in synovial material from patients with an inflammatory joint disease has also been reported (Gerard et al. 2000).

Systemic spreading of C. pneumoniae into various tissues after acute lung infection is well characterised in animal models (Moazed et al. 1998). In humans, the agent is more or less frequently found in circulating blood peripheral mononuclear cells (Smieja et al. 2002). By this means, C. pneumoniae is believed to invade a variety of tissues, and it has hence been associated with the development of several chronic diseases, including cardiovascular diseases (Leinonen & Saikku 2002), myocarditis (Wesslen et al. 1992), chronic obstructive pulmonary disease and asthma (Hahn 1999), lung cancer (Laurila et al. 1997, Koyi et al. 2001), Alzheimer’s disease (Balin et al. 1998), multiple sclerosis (Stratton et al. 2000) and reactive arthritis (Gerard et al. 2000), as already mentioned above. Unequivocal proof of direct causality between persistent C. pneumoniae and any of these diseases has not been presented yet. However, there is powerful evidence suggesting an association with cardiovascular diseases, lung complications and reactive arthritis. The associations with Alzheimer’s disease and multiple sclerosis, on the other hand, are highly controversial.

2.2 Pathogenesis of C. pneumoniae infections

The inflammatory responses elicited by chlamydia, especially after reinfections, explain the tissue damage and scarring seen in C. trachomatis infections. Chlamydial infections activate T-cell-mediated immunity, and, roughly speaking, the production of Th1 type cytokine responses and secretion of IFN-γ induced by proinflammatory IL-12 and IL-18 responses are crucial for improved recovery from chlamydial infections (Rottenberg et al. 1999, Rottenberg et al. 2000, Halme et al. 2000). On the other hand, production of a TH2 response, including anti-inflammatory IL-10 secretion, has been shown to attenuate the cell-mediated immune reactions needed for the clearance of chlamydia (Yang et al. 1996, Yang et al. 1999). The balance between these two responses or the dominance of one or the other during a chlamydia infection, leading either to clearance of infection or to pathogenic persistence, may be dependent on several factors. These may include the host’s genetic background, HLA molecules and cytokine gene polymorphism, environmental and epidemiological factors, mixed infections and the species or dose of the infecting agent. Immunity to C. pneumoniae has been reviewed by several authors, including (Surcel 2004).
The abilities of *C. pneumoniae* to infect several cell types and to disseminate into various tissues after respiratory infection are prerequisites for the various pathogenic consequences associated with *C. pneumoniae* infection. Chlamydial Hsp60 (cHsp60) is able to activate many of these cell types (Kol et al. 1999), and increasing evidence suggests that heat shock proteins play an important role in chlamydial pathogenesis (Peeling et al. 1998, Peeling et al. 1999, Lichtenwalner et al. 2004). The development of an autoimmune response against the Hsps of the host induced by bacterial Hsps due to the similar, conserved structure of these molecules has been suggested (Harboe & Quayle 1991). In an experimental model, concomitant immunisation of mice with mouse Hsp60 (mHsp60) and cHsp60 induced a potent T- and B-cell response to mHsp60, whereas immunisation with mHsp60 alone or together with highly immunogenic ovalbumin did not elicit this response (Yi et al. 1997). Molecular mimicry and an autoimmune response against self-Hsp60 have been proposed to participate in atherosclerotic development as well (Wick 2001, Lamb et al. 2003). In accordance with this, serological studies have shown antibodies to human and chlamydial Hsp60 to be risk factors for atherosclerosis (Burian et al. 2001b, Huittinen et al. 2002), colocalization of cHsp60 with human Hsp60 has been detected in atherosclerotic plaque macrophages (Kol et al. 1998), and in mice, *C. pneumoniae* inclusions lead to the development of mHsp60 autoantibodies (Erkkila et al. 2002, Erkkilä et al. 2004). In addition, immunisation with Hsp65 has also been shown to increase fatty streak formation in mice (George et al. 1999) and development of atherosclerosis in rabbits (Xu et al. 1992).

### 2.3 Animal models of *C. pneumoniae* infection

The first animal models assessing the effect of the newly discovered human pathogen 15 years ago were two studies on nonhuman primates: cynomolgus monkeys inoculated intranasally and into a conjunctival sac and nasopharynx (Holland et al. 1990b) and baboons inoculated into the conjunctiva, nasopharynx, oropharynx and trachea (Bell et al. 1989). These animals did not develop clinical signs of disease, but chlamydia was found to persist in the nasopharynx for several weeks after inoculation. In cynomolgus monkeys, chlamydia was detected in samples for prolonged periods after secondary inoculation, and partial protective immunity was seen after a third inoculation as diminished shedding of chlamydia.

*C. pneumoniae* has also been shown to infect mice (Kuo et al. 1986), and similar self-restricting infections with partial protective immunity to reinfections as in the case of nonhuman primates were later seen in murine models. The presence of viable chlamydia culturable from lung tissue is most marked for seven to ten days after primary inoculation, and *C. pneumoniae*-specific immunoglobulin class G (IgG) antibodies appear around eight to ten days after inoculation. The infection is then gradually resolved in three to five weeks, depending on the mouse strain and the infection dose used. Recovery of chlamydia from lung tissue is markedly diminished after reinfection. (Yang et al. 1993, Kaukoranta-Tolvanen et al. 1993, Kaukoranta-Tolvanen et al. 1995, Penttilä et al. 1998a). Several mouse strains have been tested, and although all strains were successfully infected, differences in the susceptibility to infection were detected: NIH/S
and Swiss Webster mice were found to be the most susceptible and uniformly infected (Yang et al. 1993, Kaukoranta-Tolvanen et al. 1993). An inoculation dose of approximately 2.7×10⁷ inclusion-forming units (IFUs) per mouse (isolate AR-39) caused sickness and occasional deaths in the mice (Yang et al. 1993), whereas a ten times lower dose (1-2×10⁶ IFUs/mouse with isolate TW-183) produced marked lung inflammation but no visible symptoms of disease (Kaukoranta-Tolvanen et al. 1993). Systemic spread of viable but not UV-inactivated chlamydia into spleen, liver and peritoneal macrophages has been detected, and chlamydia was also found in peripheral blood mononuclear cells of infected mice (Yang et al. 1995, Moazed et al. 1998). Prolonged persistence of chlamydial antigens in several tissues was detected in ApoE-deficient mice after three repeated C. pneumoniae inoculations (Moazed et al. 1997).

Typical pneumonitis developing after acute C. pneumoniae infection in animal models consists of infiltrating polymorphonuclear leucocytes in the interstitium, bronchiolar lumen and alveolar space during the first couple of days. These are then replaced by mononuclear cells during the first week of infection (Yang et al. 1994). Later, patchy consolidations of perivascular and peribronchial lymphoid and plasma cells are commonly detected, and minimal mononuclear cell infiltrates may persist for several weeks after an acute infection (Yang et al. 1993). Lung pathology was not attenuated after secondary infection despite diminished isolation counts (Kaukoranta-Tolvanen et al. 1995); in fact, the lymphoid reaction was even shown to be worsened by reinfection (Penttilä et al. 1998a). During the early course of infection, chlamydial inclusions were often seen in the cytoplasm of ciliated bronchial epithelial cells and in mononuclear cells resembling macrophages in the interstitium (Yang et al. 1994).

Rabbits were found to be susceptible to C. pneumoniae infection as well, showing similar lung inflammation in a self-resolving respiratory disease, but recovery of viable chlamydia from the lungs was more difficult (Moazed et al. 1996, Fong et al. 1997). In these studies, systemic spread of the organism from the respiratory tract to several other organs and their presence in circulating mononuclear cells were seen, as in the murine infection models.

Immune defence during primary C. pneumoniae infection in mice is dependent on T-cells, and the role of antibodies is of minor importance (Penttilä et al. 1999). Although CD8⁺ T cells seem to be more important in clearing the mouse lung infection, they are not essential alone, since only SCID mice which lack both T and B cells, CD4⁻/⁻/CD8⁻/⁻ double knockout mice (Rottenberg et al. 1999) or thymusless mice (Penttilä et al. 1999) failed to resolve the infection. Depletion of only CD4⁺ cells had no effect, and depletion of CD8⁺ cells led to a merely slight reduction in the presence of chlamydia (Penttilä et al. 1999). CD4⁺ cells in the absence of CD8⁺ cells were even shown to be harmful during the early stage of the infection (Rottenberg et al. 1999). CD8⁺ cells have also been shown to have an important role in the development of immunological memory and clearance after secondary inoculation (Penttilä et al. 1999). Recently, however, Mygind and colleagues reported a CD4⁺ T-cell response to Pmps in C57BL/6J mice, although recombinant proteins probably act differently in vivo compared to live bacteria (Mygind et al. 2004). Despite the different cells mediating the response, the predominance of the TH1-type cytokine response and especially the role of IFN-γ in resolving chlamydial murine lung and genital infections is important (Cotter & Byrne 1996, Vuola et al. 2000, Rottenberg et al. 2000, Pal et al. 2003). Based on these preliminary results, several
groups are working on the development of vaccine against *C. pneumoniae*. Production of partial protective immunity by using different chlamydial antigens and vaccine types has been reported *in vivo* in mice, but unfortunately, eradicating immunity has not been achieved (Svanholm *et al.* 2000, Murdin *et al.* 2000, Penttilä *et al.* 2000, Bandholtz *et al.* 2002, Penttilä *et al.* 2004). A vaccine consisting of several epitopes that produce protective immunity is assumed to be effective (Finco *et al.* 2005), and multiepitope DNA vaccines have recently given promising results (Pinchuk *et al.* 2005).

### 2.4 Atherosclerosis

Atherosclerosis is a complex multifactorial disease process characterized by the accumulation of lipids and fibrous elements in the large arteries, and it is the underlying cause of about 50% of all deaths in the Western societies. Early lesions of atherosclerosis, i.e. fatty streaks, can be found in the aorta as early as the first decade of life, and although not clinically significant, they are precursors of more advanced lesions. However, not all of the early fatty streaks develop further into advanced lesions. Atherosclerotic lesions may eventually grow towards the lumen, congesting the vessel and obstructing blood flow. Alternatively, a sudden rupture of the plaque may lead to haemorrhage, thrombus activity and clotting followed by complete occlusion of the artery and myocardial infarction (MI) (Ross 1993, Lusis 2000).

It has become evident that atherosclerosis is an inflammatory disease in which the endothelium plays an important role in the response to injury (Ross 1999). The aortic endothelium functions as a selectively permeable barrier between blood and tissue, and it can generate effector molecules regulating thrombosis, inflammation and vascular relaxation and contractility (Lusis 2000). According to the theory of response to injury, endothelial dysfunction in the arteries has been causally attributed to modified low-density lipoprotein (LDL) and other apolipoprotein B-containing lipoproteins, increased plasma homocysteine concentrations, hypertension, shear stress and microbes. LDL trapped into the endothelium can be modified by oxidation, lipolysis, proteolysis or aggregation, and modified LDL is thought to be the major cause of endothelial injury and altered endothelial function. The resulting abnormal state of the endothelium is therefore a consequence of injurious factors and compensatory responses to the injury. It includes altered homeostatic properties of the endothelium, i.e. increased permeability, expression of adhesive glycoproteins by endothelial cells, increased trapping of lipoproteins in the artery wall, and production of cytokines, growth factors and vasoactive molecules such as nitric oxide (NO). Adhesion molecules, chemotactic proteins and growth factors attract monocytes and T-lymphocytes to migrate into the subendothelium of the vascular wall where monocytes develop into macrophages and start accumulating modified lipids, mainly oxidised LDL, via scavenger receptors. The expression of scavenger receptors on macrophages is induced by transcription factors and cytokines such as tumour necrosis factor-α and IFN-γ. LDL ingestion leads to the formation of foam cells, which together with T-lymphocytes constitute the early fatty streaks. Growth factors and cytokines secreted from macrophages and T-cells promote the migration of smooth muscle cells (SMC) to the plaque areas, their proliferation as well as the production of extracellular...
fibrous matrix by SMCs. Furthermore, factors capable of disrupting the normal endothelial function, e.g. hypertension and elevated homocystein levels, are also able to increase proliferation, growth and extracellular matrix production of SMCs, thus affecting several stages of atherosclerotic development. Finally, advanced complex lesions consist of a fibrous cap covering the lesion and an acellular necrotic core with lipids, cell debris and calcification. Active T-lymphocytes and SMCs are also present on the sides, or “shoulders”, of the lesion, and at these sites, the fibrous material of the plaque can be broken down by cytokines such as IFN-γ produced by T-cells and matrix-degrading proteases produced by macrophages. This may lead to plaque rupture and thrombus formation. It has been shown that plaque rupture frequently occurs at the edges of the lesion areas rich in inflammatory cells, supporting the view that factors contributing to inflammation, including infections, may also promote plaque rupture and thrombosis. In fact, infections can be connected to several stages of the process described above. Inflammatory cytokines are able to increase the binding of LDL to endothelial cells as well as LDL modification, and microbial agents themselves may induce endothelial dysfunction when invading the vessel wall. Intravascular infection may also sustain inflammation by producing proinflammatory mediators, and these can, among other things, augment the expression of macrophage scavenger receptors. In addition, infection in general increases the number of circulating monocytes and lymphocytes and induces procoagulant and other haemostatic factors, thus adding to the possibility of thrombosis. (Ross 1999, Lusis 2000, Libby et al. 2002). Effects of C. pneumoniae on factors related to this process are listed later, in Table 1.

Atherosclerosis is a complex and multifactorial disease, and there are a wide variety of risk factors associated with its development. The well acknowledged traditional risk factors include male sex, age, elevated levels of LDL and reduced levels of high-density lipoprotein (HDL), hypertension, family history of premature cardiovascular disease, smoking, diabetes and metabolic syndrome. Since the recognition of the inflammatory characteristics of the disease, blood haemostatic factors, including fibrinogen and inflammatory markers such as C-reactive protein (CRP), have been added to the list. (Lusis 2000, Fruchart et al. 2004). It is quite possible that new risk factors may still emerge, infections being one candidate.

### 2.4.1 Atherosclerosis animal models

Mice are the most commonly used animals in experimental atherosclerosis studies, not least because they are easy to acquire and maintain due to their small size, and also because inbred strains are easily available. These rodents are easy to breed, and importantly, it is possible to knock out and replace endogenous genes in mice in addition to having transgenic models. (Smith 1998). Wild-type mice are normocholesterolemic, and most lipids are carried by HDL (Chapman 1986), which is known in humans to be protective against atherosclerosis. Atherosclerotic lesions do not develop spontaneously in mice, and hypercholesterolemia has to be induced either by genetic manipulation or special diets containing high levels of fat and cholesterol. The atherogenic diet used to induce hypercholesterolemia contains 15% fat, 1.25% cholesterol and 0.5% cholic acid,
and by using this diet, Paigen and co-workers found C57BL/6J mice among different inbred strains to be the most susceptible to atherosclerotic changes (Paigen et al. 1985). Lesion formation in these mice is restricted to the aortic root or aortic sinus area, and the lesions are mainly early stage fatty lesions with some invading SMCs (Smith 1998). The model is therefore suitable for studying the initiation and early stages of atherosclerosis. Moreover, the unphysiological atherosclerotic diet in itself has been found inflammatory in fatty streak-susceptible C57BL/6J mice (Liao et al. 1993).

ApoE knockout mice are the most commonly used genetically modified models in atherosclerosis research. These mice have total cholesterol levels about five times higher than those detected in wild-type mice, and they consist mainly of cholesterol ester-enriched very-low-density lipoprotein particles. (Smith 1998) The progression of lesions in ApoE-deficient mice resembles that seen in humans, with the exception that plaque rupture does not occur. However, several studies suggest that the inflammatory responses to infection are altered in these animals (Roselaar & Daugherty 1998, Laskowitz et al. 2000, de Bont et al. 2000). Other commonly used genetically modified models are LDL receptor-deficient and human ApoB-transgenic mice, which both develop large atherosclerotic lesions in response to an atherogenic diet or to a less toxic “Western diet”, which contains less cholesterol and no cholic acid (Smith 1998).

A large number of other animal species are also used in atherosclerosis research, as reviewed earlier (Narayanaswamy et al. 2000). Among these, rabbits develop both spontaneous and diet-increased atherosclerosis, while swine develop lesions whose distribution, pathogenesis and morphology resemble those in humans. For these reasons, rabbits and swine are also popular and commonly used models.

### 2.4.2 *C. pneumoniae* and atherosclerosis

The association between chronic *C. pneumoniae* infection and atherosclerosis was first suggested in 1988 by Saikku and colleagues based on a seroepidemiologic study of patients with acute MI or chronic coronary heart disease (Saikku et al. 1988). Since that, numerous seroepidemiologic and other studies have reported both positive and negative associations, some of which were reviewed by Danesh and co-workers (Danesh et al. 1997, Danesh et al. 2000, Danesh et al. 2002). Meta-analyses of prospective studies suggested a weakly positive odds ratio of marginal statistical difference for the association of *C. pneumoniae* IgA and coronary heart disease (Danesh et al. 2002), whereas no statistically significant association was found in studies on *C. pneumoniae* IgG (Danesh et al. 2000). Factors such as the high overall prevalence of *C. pneumoniae* antibodies in the older population as well as the fluctuating nature of the chlamydial antibody response show that the measurement of IgG is neither a very relevant method for assessing persistent exposure to *C. pneumoniae*, nor usable as a predictive risk factor. Most of the serological tests used in these studies have also been developed for the diagnosis of acute infections. Combination of different markers of infection, such as *C. pneumoniae* IgA antibodies, autoantibodies to Hsp60 and elevated CRP levels, has been shown to increase the risk for coronary events significantly (Huittinen et al. 2002).
Large amounts of data from in vitro cell culture studies, pathological examinations and animal models support the role of *C. pneumoniae* in atherosclerotic development. The most relevant of these studies are shown in Table 1.

Isolation of viable chlamydia from atherosclerotic plaques is a rare event, similarly as reported in the case of *C. trachomatis* and reactive arthritis: only a few studies were able to isolate chlamydia from synovial samples despite the obvious presence and viability of the organism (Villareal et al. 2002). These studies indicate that chlamydiae are present in the tissue in an aberrant persistent form and are very difficult to culture. Several studies have also failed to detect *C. pneumoniae* in plaques by PCR (Weiss et al. 1996, Ong et al. 2001, Maraha et al. 2004), and the overall range of positive research findings varies from 0 to 100% (Kalayoglu et al. 2002). The PCR methods used in the detection of *C. pneumoniae* have not been standardised, and their sensitivities can be highly variable (Apfalter et al. 2001). Besides the methodological defects, the PCR inhibitors present in atheromatous tissue as well as the patchy distribution of chlamydia in plaque may have contributed to the negative results (Leinonen & Saikku 2002, Kalayoglu et al. 2002.). Although *C. pneumoniae* infections promote atherosclerotic lesions in animal models, infection itself does not seem to affect the serum lipid profiles in mice (Hu et al. 1999, Blessing et al. 2001), and only transient changes have been reported (Tiirola et al. 2002). Two infection models using ApoE-deficient mice have failed to detect increased lesion development after *C. pneumoniae* inoculations (Caligiuri et al. 2001, Aalto-Setälä et al. 2001). It is also of note that infection of mice with *C. trachomatis* did not increase atherosclerotic lesions, thus indicating the species-specific effect of *C. pneumoniae* (Hu et al. 1999, Blessing et al. 2000b).
Table 1. Studies supporting the role of C. pneumoniae infection in the development of atherosclerosis.

<table>
<thead>
<tr>
<th>Effect of C. pneumoniae in in vitro studies and in animal models and detection in atheromas</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro studies</strong></td>
<td></td>
</tr>
<tr>
<td>C. pneumoniae is able to infect all the cell types detected in atherosclerosis, i.e. coronary artery endothelial cells, smooth muscle cells and monocytes/macrophages.</td>
<td>Godzik et al. 1995, Gaydos et al. 1996, Kaukoranta-Tolvanen et al. 1996b</td>
</tr>
<tr>
<td>Induced foam cell formation of infected macrophages by chlamydial LPS.</td>
<td>Kalayoglu &amp; Byrne 1998, Kalayoglu et al. 1999</td>
</tr>
<tr>
<td>Increased expression of adhesion molecules, chemokines and procoagulant factors in infected epithelial cells.</td>
<td>Molestina et al. 1999, Kalayoglu et al. 2001</td>
</tr>
<tr>
<td>Induced production of inflammatory cytokines by infected cells.</td>
<td>Miller et al. 2000, Netea et al. 2000, Blessing et al. 2002</td>
</tr>
<tr>
<td>Increased adherence of infected monocytes to endothelium and transendothelial migration.</td>
<td>Dechend et al. 1999, Summersgill et al. 2000</td>
</tr>
<tr>
<td>Production of matrix-degrading metalloproteinases enhanced by infection.</td>
<td>Kol et al. 1998, Vehmaan-Kreula et al. 2001</td>
</tr>
<tr>
<td>Presence in plaques</td>
<td></td>
</tr>
<tr>
<td>C. pneumoniae detected in about 50% of atheromatous tissue by PCR, in situ hybridisation and immunocytochemistry but generally not in healthy tissue.</td>
<td>Reviewed in Kalayoglu et al. 2002</td>
</tr>
<tr>
<td>Transmission electron microscopic detection of chlamydia in coronary artery atheromas but not in normal tissue.</td>
<td>Shoret et al. 1992, Kuo et al. 1993</td>
</tr>
<tr>
<td>Successful isolation of viable C. pneumoniae from atherosclerotic tissue</td>
<td>Ramírez 1996, Jackson et al. 1997, Maas et al. 1998</td>
</tr>
<tr>
<td><strong>Animal models</strong></td>
<td></td>
</tr>
<tr>
<td>Early atherosclerotic changes induced after primary inoculation in New Zealand White rabbits.</td>
<td>Fong et al. 1997</td>
</tr>
<tr>
<td>Induction of early atherosclerotic lesions in New Zealand White rabbits fed a normal chow after single or repeated inoculations.</td>
<td>Laritan et al. 1997, Fong et al. 1999a, Fong et al. 1999b, Fong et al. 2002</td>
</tr>
<tr>
<td>Increased maximal intimal thickness of the thoracic aortas in New Zealand White rabbits fed a chow with 0.25% cholesterol.</td>
<td>Muhlein et al. 1998</td>
</tr>
<tr>
<td>Induction of inflammatory changes in the heart and aorta of normocholesterolemic C57BL/6J and BALB/c mice after repeated inoculations.</td>
<td>Blessing et al. 2000a, Burian et al. 2001a</td>
</tr>
<tr>
<td>Increased lesions in C57BL/6J mice fed an atherogenic diet after repeated inoculations.</td>
<td>Blessing et al. 2001</td>
</tr>
<tr>
<td>Accelerated formation of complex atherosclerotic lesions in ApoE-Leiden mice fed an atherogenic diet after two inoculations.</td>
<td>Ezzahiri et al. 2002</td>
</tr>
<tr>
<td>Endothelial dysfunction induced by single or repeated inoculations in ApoE-deficient mice, pigs and C57BL/6J mice.</td>
<td>Liuba et al. 2000, Liuba et al. 2003</td>
</tr>
<tr>
<td>Increased atherosclerosis in C57BL/6J and in eNOS−/− and iNOS−/− mice fed an atherogenic diet after repeated inoculations.</td>
<td>Chezebro et al. 2003</td>
</tr>
<tr>
<td>Increased adherence of C. pneumoniae-infected macrophages to the carotid artery wall in nonatherosclerotic mice</td>
<td>May et al. 2003</td>
</tr>
<tr>
<td>Increased subendothelial lipid accumulation in normocholesterolemic C57BL/6J mice fed a cholesterol-supplemented diet.</td>
<td>Erkkilä et al. 2004</td>
</tr>
</tbody>
</table>

1lipopolysaccharide, 2apolipoprotein E knockout, 3low density lipoprotein, 4endothelial nitric oxide synthase, 5inducible nitric oxide synthase
2.4.3 Other microbes associated with atherosclerosis and cardiovascular diseases

Besides *C. pneumoniae*, other bacteria and several viruses have been associated with the development of atherosclerosis as well. Most published studies have focused on *Helicobacter pylori*, cytomegalovirus, herpes simplex virus and enteroviruses (Roivainen et al. 1998, Roivainen et al. 2000) as well as periodontal pathogens (Mattila 2003). In addition to these, hepatitis A and picornaviruses have been brought up, as reviewed in (Leinonen & Saikku 2002). The majority of these studies are seroepidemiologic and suffer from methodological problems (Danesh et al. 1997). As with *C. pneumoniae*, the fluctuating quality and high prevalence of antibody responses in the population weaken the power of seroepidemiological studies to detect the association between the microbe and atherosclerosis. Nucleic acids or antigens of *H. pylori* (Ameriso et al. 2001, Kowalski 2001), periodontal pathogens (Haraszthy et al. 2000, Fiehn et al. 2005), enterovirus (Kwon et al. 2004) and cytomegalovirus (Hendrix et al. 1989) have been found in human atherosclerotic plaques. In animal models, cytomegalovirus and *Porphyromonas gingivalis*, a periodontal pathogen, have been shown to increase atherosclerotic lesions in ApoE-deficient mice (Burnett et al. 2001, Vliegen et al. 2002, Gibson et al. 2004). Recent data have also suggested new mechanisms of action whereby infections may contribute to atherosclerosis. Periodontitis has been shown to increase macrophage activation via LPS (Pussinen et al. 2004b) and to cause atherogenic changes in HDL metabolism (Pussinen et al. 2004a). Further, treatment of periodontitis with both mechanical and antimicrobial therapy significantly improved endothelial function measured by flow-mediated dilatation of the brachial artery and decreased CRP concentrations in periodontitis patients (Seinost et al. 2005).

Besides the pathogens mentioned above, occasional reports suggesting an association of human immunodeficiency virus as well as Epstein-Barr, coxsackie B and measles viruses with atherosclerotic development have been published, but the evidence of these pathogens as the sole causative agents in atherosclerosis is faint (Morre et al. 2000). Nonetheless, all the infectious agents referred above may together contribute to atherosclerotic development. It has been shown that people infected with multiple pathogens and with elevated inflammatory markers (mainly CRP) have the greatest relative risk for coronary artery disease, and based on this, Zhu and colleagues proposed the concept of “pathogen burden” as a risk factor for atherosclerosis (Zhu et al. 2000). The finding that non-specific stimulation of the innate immune system by LPS accelerates cholesterol-induced atherosclerosis in rabbits lends support to this concept (Lehr et al. 2001).

2.5 Antibiotic treatment of chlamydial infections

Treatment of chlamydial infections may be problematic since treatment failures are often reported, possibly due to the ability of chlamydiae to trigger persistent infections not responsive to antimicrobial treatment (Oriel & Ridgway 1982, Kutlin et al. 1999, Gieffers
et al. 2001, Baltch et al. 2004, Reveneau et al. 2005). As mentioned earlier, antibiotics used for treatment may in themselves also cause persistence of the infection (Dreses-Werringloer et al. 2000, Gieffers et al. 2004). In general, chlamydiae are susceptible to tetracyclines (Welsh et al. 1992, Miyashita et al. 1997), macrolides (Welsh et al. 1992, Miyashita et al. 1997), fluoroquinolones (Miyashita et al. 1997) and rifampin (Ridgway et al. 1978), but less susceptible to clindamycin (Walsh et al. 1987) and cloramphenicol (Kuo et al. 1977) and resistant to aminoglycosides (gentamycin) (Wentworth 1973, Pearlman et al. 1990) and glycopeptides (vancomycin) (Ridgway et al. 1978). Some C. trachomatis strains are also partially susceptible to sulphonamides (Kuo et al. 1977), whereas C. pneumoniae strains are not (Kuo & Grayston 1988). The inconsistent effects of penicillin and related compounds were discussed earlier in chapter 2.1.1: these are able to prevent the production of infective chlamydia but can neither block the formation of chlamydial inclusions nor kill the bacteria in cell cultures (Kuo & Grayston 1988). Chlamydiae have three different penicillin-binding proteins, and at least in the case of C. trachomatis, benzylpenicillin is able to bind all these proteins efficiently (Storey & Chopra 2001). C. trachomatis has been shown to be more susceptible to penicillin derivatives, but not to cephalosporins (Hammerschlag & Gleyzer 1983), than C. pneumoniae (Kuo & Grayston 1988), and a wide-spectrum penicillin-derivative drug, amoxicillin, has been used to treat chlamydial infections in pregnant women (Miller & Martin 2000).

Azithromycin treatment given as a single dose of 1 g is considered the first-line treatment of sexually transmitted chlamydial disease and trachoma, but the traditional 7-day course of doxycycline and topical therapy with tetracycline in the management of active trachoma are also used (Martin et al. 1992, Bailey et al. 1993, Lau & Qureshi 2002). The high cellular and tissue concentrations as well as the long half-life of azithromycin in tissue allow single dosage and therefore better compliance as well (Gordon & Blumer 2004).

In vitro susceptibilities of C. pneumoniae to different antibiotics are presented in Table 2. Macrolides and tetracyclines are the most commonly used drugs in the treatment of acute chlamydial infections. Fluoroquinolones, rifampin and, most recently, ketolides have also been found to be effective. Still, there are no generalized guidelines, such as those for C. trachomatis treatment, and data are limited on the treatment of acute lung infections caused by chlamydia. Prolonged therapy from ten days to two weeks, similar to that of psittacosis, with either macrolide or tetracycline has been recommended (Grayston et al. 1990). A study reporting pneumonia treatment in children with either azithromycin for five days or erythromycin or amoxicillin-clavulanate for 10 days showed 83% eradication as determined by culture from patients. Despite the persistence of chlamydia in some cases, all patients were clinically cured with these treatments (Roblin & Hammerschlag 1998b). In another study of Japanese adults with prolonged cough, some patients were clinically improved and PCR positivity from nasopharyngeal swabs disappeared after only two or three courses of clarithromycin treatment for two weeks (Miyashita et al. 2003b). Therefore, good in vitro susceptibility results do not always agree with in vivo treatment.
Table 2. In vitro susceptibility of *C. pneumoniae* to different antimicrobial agents, measured as minimal inhibitory concentration (MIC) of the antibiotic in cell cultures.

<table>
<thead>
<tr>
<th>Antibiotic group and way of function</th>
<th>Specific drug</th>
<th>MIC range (µg/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-lactam antibiotics – prevents the formation of peptidoglycan in the bacterial cell wall</td>
<td>Penicillin</td>
<td>&gt;100</td>
<td>Kuo &amp; Grayston 1988</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>&gt;128</td>
<td>Pudjiatmoko <em>et al.</em> 1998</td>
</tr>
<tr>
<td>Sulfonamides - block folic acid synthesis in bacteria</td>
<td>Sulfisoxazole</td>
<td>&gt;400</td>
<td>Kuo &amp; Grayston 1988</td>
</tr>
<tr>
<td></td>
<td>Sulgemethoxazole</td>
<td>&gt;500</td>
<td>Chirgwin <em>et al.</em> 1989</td>
</tr>
<tr>
<td>Tetracyclines – bind the ribosomal 30S subunit and prevent accommodation of tRNAs to ribosomes</td>
<td>Tetracycline</td>
<td>0.05-0.1</td>
<td>Kuo &amp; Grayston 1988</td>
</tr>
<tr>
<td></td>
<td>Doxycycline</td>
<td>0.01-0.4</td>
<td>Gnarpe <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>Minocycline</td>
<td>0.016-0.031</td>
<td>Miyashita <em>et al.</em> 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03-0.06</td>
<td>Donati <em>et al.</em> 1999</td>
</tr>
<tr>
<td>Macrolides – bind the bacterial 50S ribosomal subunit and prevent translocation of tRNAs</td>
<td>Azithromycin</td>
<td>0.125-0.5</td>
<td>Welsh <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>0.01-0.05</td>
<td>Gnarpe <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.125-1.0</td>
<td>Welsh <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>Clarithromycin</td>
<td>0.015-0.03</td>
<td>Chirgwin <em>et al.</em> 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.031-1.0</td>
<td>Welsh <em>et al.</em> 1996</td>
</tr>
<tr>
<td></td>
<td>Roxithromycin</td>
<td>0.063-0.125</td>
<td>Niki <em>et al.</em> 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.063-0.25</td>
<td>Miyashita <em>et al.</em> 1997</td>
</tr>
<tr>
<td>Fluoroquinolones – bind bacterial DNA gyrase (topoisomerase II) and inhibit its activity</td>
<td>Ciprofloxacin</td>
<td>1.0</td>
<td>Chirgwin <em>et al.</em> 1989</td>
</tr>
<tr>
<td></td>
<td>Levofloxacin</td>
<td>1.0-2.0</td>
<td>Miyashita <em>et al.</em> 1997</td>
</tr>
<tr>
<td>Ketolides – bind bacterial 50S ribosomal subunits and prevent translocation of tRNAs</td>
<td>Telithromycin</td>
<td>0.015-2.0</td>
<td>Roblin &amp; Hammerschlag 1998a</td>
</tr>
<tr>
<td></td>
<td>Cethromycin</td>
<td>0.031-0.25</td>
<td>Miyashita <em>et al.</em> 2003a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.016-0.031</td>
<td>Miyashita <em>et al.</em> 2003a</td>
</tr>
<tr>
<td>Rifamycin derivatives – inhibit the function of bacterial RNA polymerase</td>
<td>Rifampin</td>
<td>0.0075-0.031</td>
<td>Freidank <em>et al.</em> 1999</td>
</tr>
<tr>
<td></td>
<td>Rifalazil</td>
<td>0.00125</td>
<td>Roblin <em>et al.</em> 2003</td>
</tr>
</tbody>
</table>

*Susceptibilities of *C. pneumoniae* strains for a wide variety of fluoroquinolones have been tested. For more information about different compounds, see references (Miyashita *et al.* 1997, Hammerschlag 2000).

Macrolide antibiotics, especially semi-synthetic erythromycin A derivatives (e.g. clarithromycin and roxithromycin), have been shown to possess several anti-inflammatory activities (Scaglione & Rossoni 1998, Labro 1998, Ianaro *et al.* 2000). Several studies have also shown fluoroquinolones to be anti-inflammatory both in models with epithelial cells (Uriarte *et al.* 2004) and in models with macrophages (Azuma *et al.* 1999) and in vivo (Jimenez-Valera *et al.* 1995). These properties may influence the outcome of the treatment.
2.5.1 Animal models for C. pneumoniae treatment

The treatment of acute C. pneumoniae infection in murine models has shown that the antimicrobial agents tested are efficient in abolishing the presence of viable chlamydia in lung tissue. Macrolides and fluoroquinolones also decrease lung inflammation effectively, but nevertheless, persistence of chlamydial antigens in lung tissue despite treatment is often reported. In the earliest studies, a single dose of azithromycin or three days of doxycycline similarly accelerated the clearance of viable chlamydia from mouse lung tissue compared to placebo-treated controls (Malinverni et al. 1995b). Despite this, no differences in lung inflammation between the treatment and control groups were seen, and chlamydial DNA was frequently recovered from culture-negative lungs during 14 days after inoculation. A single dose of azithromycin combined with a short course of rifampin was then evaluated after acute infection in comparison to azithromycin treatment alone: the combination treatment showed superior effects in chlamydial clearance (Wolf & Malinverni 1999). To assess the effect of longer treatment with combined antibiotics on the clearance and persistence of chlamydia, treatments with azithromycin plus rifampin vs. amoxicillin and placebo-treated mice were tested (Bin et al. 2000). Combination treatment abolished the culture positivity of the organisms in lung tissue very effectively, but about half of the mice remained positive for chlamydial antigens at two months after inoculation. In this study, the number of lung mononuclear cell infiltrates was also significantly decreased by azithromycin plus rifampin treatment, whereas no differences in this respect were detected between amoxicillin treatment and the control group. The lung inflammation in amoxicillin-treated mice was not alleviated during the 60 days of follow-up. In a treatment study by another group using mice immunosuppressed by cyclophosphamide to produce a longer-lasting acute infection resembling clinical illness, oral doses of doxycycline, erythromycin, azithromycin, amoxicillin-clavulanate, ofloxacin and ciprofloxacin for seven days were tested (Masson et al. 1995). No significant differences in the presence of viable chlamydia or lung inflammation were detected between the different treatments, and none of the treatments eradicated the organism from all animals. The combination of amoxicillin and clavulanate was also shown to be effective in vivo, despite the poor potency of β-lactams reported in several in vitro studies, as discussed above. The long-term persistence of chlamydia after these treatments was, unfortunately, not studied in this work. Another study using leukopenic mice (leukopenia induced by cyclophosphamide) examined the effects of oral administration of sparfloxicin, ofloxacin, tosufloxacin, minocycline and clarithromycin on the survival rate and lung inflammation of inoculated mice (Nakata et al. 1994). Minocycline, clarithromycin and sparfloxicin were the most effective drugs in vitro, and sparfloxicin was also the most potent treatment in vivo. In addition to resulting in high survival rates, it almost completely abolished the neutrophil and lymphocyte infiltrations into lung tissue at a relatively low dose (3 mg/kg) of the drug.

A very recent study assessed the effect of glucocorticoid treatment combined with azithromycin in a murine model (Caronzolo et al. 2004). Since immunosuppressive cortisone administration during persistent infection has been shown to reactivate chlamydia infection in mouse lungs (Malinverni et al. 1995a, Laitinen et al. 1996), this, followed by antimicrobial treatment, might be useful in eradicating persistent chlamydial
infection. Caronzolo et al. used these drugs concurrently for four days, starting four days after inoculation. The combination was significantly more active in reducing chlamydia in lungs compared to azithromycin alone.

In addition, treatment of rabbits with azithromycin after three repeated C. pneumoniae inoculations decreased significantly the infection-induced maximal intimal thickness of the thoracic aortas and the plaque area index (Muhlestein et al. 1998). In other studies with rabbits, Fong and colleagues compared early azithromycin (Fong et al. 1999a) or clarithromycin (Fong et al. 2002) treatments started five days after an initial inoculation to delayed treatment started two weeks after a third C. pneumoniae inoculation. Early treatment had a superior effect compared to delayed treatment in decreasing the early signs or lesions of atherosclerosis with both antibiotics tested. Delayed treatment with azithromycin even seemed to increase the presence of chlamydial antigens in the aortic segments (Fong et al. 1999a). In another set of experiments with rabbits fed a cholesterol-enriched diet, the anti-inflammatory effect of clarithromycin was tested on the development of atherosclerotic lesions without chlamydial infection. Only a mild reduction in the atherosclerotic changes was observed, and the beneficial effects of clarithromycin were concluded to be mostly due to the antimicrobial effect (Fong et al. 2002). Partly contradictory to the results obtained from rabbit models, azithromycin did not decrease the progression of lipid lesions in ApoE-deficient mice when the treatments were started after the induction of persistent infection with repeated inoculations (Rothstein et al. 2001, Blessing et al. 2005).

### 2.5.2 Human studies with antibiotic treatment

The first two small-scale antibiotic treatment trials in coronary heart disease patients with azithromycin or roxithromycin (Gupta et al. 1997, Gurfinkel et al. 1997) gave promising results. Since these, several large-scale intervention studies, some with markedly longer treatment periods, have failed to show a persistent beneficial effect of treatment on the cardiovascular end point events. The most important intervention studies with results available are presented in Table 3, modified and further augmented after (Higgins 2003) and (Andraws et al. 2005).
Table 3. Randomised controlled trials of antibiotics in atherosclerotic vascular diseases. (Modified after Higgins 2003, Andraws et al. 2005)

<table>
<thead>
<tr>
<th>Trial (Reference)</th>
<th>Study population</th>
<th>Treatment</th>
<th>Primary end point</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of patients with post myocardial infarction (MI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St. Georges Hospital, UK (Gupta et al. 1997)</td>
<td>220 male patients, treatment of Cpn-seropositive (IgG ≥ 1:64)</td>
<td>Azithromycin for three of six days; follow-up for 18 months</td>
<td>Nonfatal MI, unstable angina, cardiovascular death</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>WIZARD (O’Connor et al. 2003)</td>
<td>7724 patients (83% male), Cpn IgG ≥ 1:16</td>
<td>Azithromycin for three months; mean follow-up 2.5 years</td>
<td>Recurrent MI, death, revascularization, angina/ischemia</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>CROAATS (Reiner 2003)</td>
<td>337 patients with two Cpn-seropositive serum samples (IgG ≥ 1:20)</td>
<td>Azithromycin for three days, starting at days 1, 10 and 20; follow-up for 18 months</td>
<td>Cardiac death, MI, revascularization, hospitalised angina</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>Treatment of patients with coronary artery disease (CAD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACADEMIC (Muhlestein et al. 2000)</td>
<td>302 patients seropositive to Cpn (IgG ≥ 1:16)</td>
<td>Azithromycin weekly for three months; follow-up for 2 years</td>
<td>Cardiac death, cardiac arrest, nonfatal MI, stroke, unstable angina, revascularization</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>ACES (Grayston et al. 2005)</td>
<td>4012 patients with CAD</td>
<td>Azithromycin weekly for one year; mean follow-up 4 years</td>
<td>Cardiac death, nonfatal MI, unstable angina and revascularization</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>Treatment of patients with acute coronary syndromes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROXIS (Gurfitinkel et al. 1999)</td>
<td>202 patients with unstable angina or non-Q-wave MI</td>
<td>Roxithromycin for 30 days; follow-up for six months</td>
<td>Cardiac death, MI, severe recurrent ischemia</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>Siriraj Hospital, Thailand (Lorawatta et al. 2001)</td>
<td>94 patients with acute coronary syndrome</td>
<td>Roxithromycin for 30 days, follow-up for three months</td>
<td>Cardiac death, unplanned revascularization, recurrent angina/MI</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>STAMINA (Stone et al. 2002)</td>
<td>325 patients with MI or unstable angina</td>
<td>Amoxicillin or azithromycin + metronidazole + omeprazole for seven days; follow-up for one year</td>
<td>Cardiac death or readmission with acute coronary artery syndrome</td>
<td>Positive</td>
</tr>
<tr>
<td>CLARIFY (Siniiso et al. 2002)</td>
<td>148 patients with unstable angina or non-Q-wave MI</td>
<td>Clarithromycin for 3 months; mean follow-up 555 days</td>
<td>Death, MI, unstable angina</td>
<td>Positive</td>
</tr>
<tr>
<td>AZACS (Cereck et al. 2003)</td>
<td>1439 patients with acute MI or unstable angina</td>
<td>Azithromycin for four days; follow-up for six months</td>
<td>Death, recurrent MI or ischemia needing revascularization</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>ANTIBHO (Zahn et al. 2003)</td>
<td>872 patients with acute MI</td>
<td>Roxithromycin for six weeks; follow-up for one year</td>
<td>Total mortality</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>PROVE-IT (Cannon et al. 2005)</td>
<td>4162 patients with recent acute coronary syndrome and total cholesterol level of 150-240 mg/dl</td>
<td>Gatifloxacin with pravastatin or atorvastatin, ten months for two years = follow-up</td>
<td>Death, stroke, MI, hospitalisation with angina, revascularation</td>
<td>No significant reduction</td>
</tr>
<tr>
<td>Treatment of patients with post-coronary stenting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISAR-3 (Neumann et al. 2001)</td>
<td>1010 patients with successful coronary stenting</td>
<td>Roxithromycin for 28 days; follow-up for one year</td>
<td>Frequency of restenosis and target vessel revascularization</td>
<td>Negative overall, positive in patients with Cpn IgG ≥ 1:512</td>
</tr>
</tbody>
</table>

*Cpn, C. pneumoniae; IgG, immunoglobulin G*
There are also some intervention studies on peripheral atherosclerotic vessel disease. Treatment of *C. pneumoniae* seropositive men with roxithromycin for one month prevented the progression of peripheral arterial occlusive disease during a follow-up period of 2.7 years (Wiesli *et al.* 2002). Another study reported the effect of 30 days of roxithromycin therapy on the progression of intima-to-media thickness (IMT) of the common carotid artery in patients with ischemic stroke (Sander *et al.* 2002). Follow-up was continued for two years, and during this time patients with *C. pneumoniae* IgG antibodies (≥1:64) showed significantly decreased IMT. However, IMT progression was shown to increase to similar values as before the treatment during the third and fourth follow-up years, thus showing the effect of the treatment to be transient (Sander *et al.* 2004). Two other smaller-scale studies assessed the effect of doxycycline for three months (Mosorin *et al.* 2001) or that of roxithromycin for 28 days (Vammen *et al.* 2001) on the growth of abdominal aortic aneurysms. Both studies reported significant reduction in aneurysm growth during the follow-up of 18 months. A third study reported the effect of doxycycline treatment for six months on small asymptomatic abdominal aortic aneurysms (Baxter *et al.* 2002). No significant change in the aneurysm diameter, however, was found after the treatment period compared to the baseline levels.

Besides the clinical cardiovascular end points, some of the studies listed in Table 3 as well as other studies have reported the effect of antimicrobial treatment on inflammatory markers (Anderson *et al.* 1999, Stone *et al.* 2002), other coronary risk factors, including fibrinogen levels (Sinisalo *et al.* 1998, Torgano *et al.* 1999, Stone *et al.* 2002), and endothelial function (Parchure *et al.* 2002). The effects of treatments on suppressing inflammatory factors have generally been positive, although a decrease in CRP was not always seen. Treatment of patients with hypercholesterolemia and previous coronary bypass with doxycycline for four months had no significant effects on coronary risk factors (Sinisalo *et al.* 1998), while some other studies have reported positive effects on fibrinogen levels (Torgano *et al.* 1999, Stone *et al.* 2002). Variable results on the effect of antibiotic treatment on endothelial function in humans have also been reported. Parchure and co-workers reported five weeks of treatment with azithromycin to induce significant improvement in flow-mediated dilatation of the brachial artery and significant reductions in E-selectin and von Willebrand factor levels (Parchure *et al.* 2002). On the other hand, Sinisalo and colleagues found no effects of four months of doxycycline treatment on the basal NO production measured as forearm blood flow responses to a nitric oxide synthase (NOS) antagonist (Sinisalo *et al.* 1998).

As reviewed above, chronic or persistent chlamydia infection does not respond to antimicrobial treatment, and this is in accordance with the results from the published intervention trials (Higgins 2003, Andraws *et al.* 2005, Danesh 2005). All of the really large-scale intervention studies with long follow-up times, regardless of the antibiotic used, were ultimately negative. It can be pointed out, however, that the trials treating acute coronary syndrome patients where the antibiotic treatment was really started within 48 hours after admission into hospital were among the studies reporting positive treatment outcomes (Gurfinkel *et al.* 1997, Stone *et al.* 2002, Sinisalo *et al.* 2002). These results together with the treatment trials of progressive abdominal aortic aneurysms showing positive outcomes (Mosorin *et al.* 2001, Vammen *et al.* 2001) suggest that, if given in an acute, progressive state, antibiotic treatment might have positive effects. Treatment of chronic *C. pneumoniae* infections with multiple antibiotics has been
suggested as well, since synergistic effects of antibiotics have been reported \textit{in vitro} (Freidank \textit{et al.} 1999, Dreses-Werringloer \textit{et al.} 2001). Yet, treatment of mice with azithromycin and rifampin together for seven days, starting two days after acute inoculation, was not able to prevent the persistence of chlamydial antigens detected by PCR and immunocytochemistry (Bin \textit{et al.} 2000). Thus, the present data imply that neither chronic chlamydia infections nor advanced atherosclerotic disease can be treated with antimicrobial drugs.

\section*{2.6 Antichlamydial potential of other compounds}

\subsection*{2.6.1 Flavonoids}

Plant polyphenolic compounds, such as flavonoids, are important metabolites in defence against radiation and pathogenic micro-organisms and are present in almost all foods of plant origin, especially teas, onion, apple, berries and red wine (Hertog \textit{et al.} 1992, Beecher 2003). There is evidence to suggest that these compounds may protect from cardiovascular diseases (Reed 2002, Aviram \textit{et al.} 2002) and cancer (Ren \textit{et al.} 2003). The low rate of coronary heart disease in France compared with the other developed countries with comparable dietary intake and high intake of saturated fat has been called the “French paradox”, and the antioxidant potential of red wine polyphenols has been suggested to explain this paradox (Hayek \textit{et al.} 1997). The suggested effects of phenolic compounds include, as mentioned, their antioxidant properties (Aruoma \textit{et al.} 1993, Kumar \textit{et al.} 2003), modulation of drug-metabolising enzymes such as cytochrome P450 (Huynh & Teel 2002), effects on platelet aggregation (Pace-Asciak \textit{et al.} 1995) and the immune system and antiviral and antibacterial activities (Kaul \textit{et al.} 1985, Cowan 1999).

In previous cell culture studies, several plant-derived phenolic compounds have been shown to possess strong antichlamydial properties \textit{in vitro} (Vuorela \textit{et al.} 2004). The most potent of the studied compounds were luteolin from the group of flavones (MIC 8.8 \mu M), docedyl, methyl and octyl gallates, which belong to the group of alkyl gallates (MICs 18, 29 and 29 \mu M, respectively), myricetin from the flavonol group (MIC 29 \mu M) and another flavone, acacetin (MIC 29 \mu M) (Alvesalo \textit{et al.} in press). Yamazaki and co-workers also found several tea polyphenols to have antichlamydial activity \textit{in vitro} against both \textit{C. trachomatis} and \textit{C. pneumoniae} (Yamazaki \textit{et al.} 2003, Yamazaki \textit{et al.} 2005).

Alkyl gallates are synthetic derivatives of gallic acid and commonly used as antioxidant additives in various foodstuffs (Aruoma \textit{et al.} 1993). Similarly to several flavonoid compounds, alkyl gallates also have wide antibacterial properties (Kubo \textit{et al.} 2004). Quercetin, the most widely studied flavonoid in the group of flavonols, and luteolin are both ubiquitous compounds and microbicidal, as shown in several studies (Kaul \textit{et al.} 1985, Rauha \textit{et al.} 2000, Xu & Lee 2001). All these three compounds have also been shown to inhibit phosphorylation cascades and proinflammatory cytokine and chemokine production either \textit{in vitro} or in animal models (Murase \textit{et al.} 1999, Xagorari \textit{et al.} 2001, Kotanidou \textit{et al.} 2002, Cho \textit{et al.} 2003).
Pharmacokinetics and bioavailability of polyphenolic compounds vary considerably between the different groups of flavonoids and also between the different forms of a given compound. Most flavonoids are present glycosylated in food, and in the case of quercetin, only the glycosylated forms are absorbed, whereas no absorption of the free, aglycone form has been detected. Yet, only very few phenolic compounds have been thoroughly studied, especially in regard of pharmacokinetics in humans, and although a number of studies reporting plasma concentrations are available, the tissue distribution of flavonoids after intake in humans is not known. (Manach et al. 2004).

2.6.2 Cathelicidin peptides

Defensins and cathelicidins are proteins that are stored in mammalian leucocytes, and cathelicidins carry divergent antimicrobial peptides at the C-terminus of their structure. These peptides, which become active when released from cathelicidin proteins, display a remarkable variety of sizes, sequences and structures (Gennaro & Zanetti 2000). Most of the cathelicidin-derived peptides exert potent antimicrobial activity, covering a range of organisms including bacteria, fungi and enveloped viruses. In addition, they are able to bind to LPS and neutralize its effects (Ciornei et al. 2005). These features make this family of peptides good candidates in view of therapeutic use. The most promising ones are currently under evaluation as potential novel anti-infectives, and synthetic variants are in an advanced stage of development for specific clinical applications (Zanetti et al. 2002). A very recent study assessed the in vitro activity of six cathelicidin peptides against 25 chlamydial strains, including five C. pneumoniae strains (Donati et al. 2005). Five of the studied compounds required concentrations higher than 80 µg/ml to reduce ≥50% of chlamydial inclusions, whereas one compound, SMAP-29, proved to be the most active peptide by causing a similar reduction at a concentration of 10 µg/ml. This peptide was also the most active against C. trachomatis, and studies in animal models were suggested to test its in vivo effects. Previously, Yasin and co-workers have studied the effects of both defensins and cathelicidin peptides (protectins) on C. trachomatis infections in vitro, and they found especially cathelicidins to be potent in inactivating chlamydia (Yasin et al. 1996). In addition, altered inclusion morphology of chlamydial particles with amorphous structure and one visible cell membrane were reported in electron microscopy.

2.6.3 Statins

Statins are the most potent class of drugs to reduce serum cholesterol levels. These drugs inhibit 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme crucial to cholesterol synthesis, and statin treatment reduces plasma levels of LDL cholesterol effectively. Several clinical trials with statins have demonstrated a marked decrease in cardiovascular risk. Yet, the improvement in cardiovascular end points in those trials was superior to estimations of the effect on lowering LDL cholesterol. Recent observations using in vivo and in vitro models of atherosclerosis have shown statins to
have potential for the manipulation of various cellular functions via inhibition of the mevalonate pathway: the inhibitory effects of statins on monocyte-endothelial interaction suggest an effect on inflammation. (Danesh et al. 2003, Arnaud & Mach 2005). In addition, statins have been shown to reduce the intracellular growth of *Salmonella enterica* *in vitro* and *in vivo* (Catron et al. 2004) and to attenuate the replication of *C. pneumonias* in cell culture (Kothe et al. 2000). Erkkilä and co-workers have studied the effects of simvastatin treatment on *C. pneumoniae* lung infection, inflammation and serum lipids in a mouse model (Erkkilä et al. 2005). Simvastatin was shown to decrease viable chlamydial counts and to increase inflammatory cell infiltrates in lung tissue, indicating both antichlamydial and proinflammatory effects during acute chlamydial infection, thus supporting the versatile functions of statins.
3 Aims of the study

The objectives of the present studies were threefold. One of the aims was to investigate the effects of primary and repeated *C. pneumoniae* inoculations on the development of acute and chronic lung infection and inflammatory responses in a wild-type mouse model. The second aim was to assess the early atherosclerotic changes induced by *C. pneumoniae* infections in this animal model, and finally, the third aim was to elucidate the effects of treatments with antibiotics and phenolic compounds on the pathologic sequelae induced by these infections.
4 Materials and methods

4.1 *C. pneumoniae* isolate

*C. pneumoniae* isolate Kajaani 7 (K7), a Finnish epidemic strain (Ekman et al. 1993) grown in HL cells, was used in the studies. Infected cells were harvested with sterile glass beads and ultrasonically disrupted. Cell debris was separated with low-speed centrifugation, followed by sonication and two cycles of high-speed centrifugation through Urografin gradients to purify chlamydial particles. Finally, the pellet obtained was resuspended in sucrose-phosphate-glutamic acid (SPG) buffer for storage. The number of viable organisms in the purified stock as IFUs/ml was determined by culture in HL cells. To do this, HL cells were infected with tenfold dilutions of the stock using centrifugation at 490xg for 1 h and incubated at 35°C under 5% CO₂ for 72 h. The culture medium (RPMI-1640) contained 7% fetal bovine serum, 1% L-glutamate, 20 μg/ml streptomycin and 0.5 μg/ml cycloheximide. After incubation, the cells were washed with phosphate-buffered saline (PBS) solution and fixed for 10 minutes with methanol. The fixed cells were stained with *Chlamydia* genus-specific monoclonal antibody conjugated to fluorescein isothiocyanate (Pathfinder®, Sanofi Diagnostics Pasteur, France). Inclusions in at least three different dilutions were counted to calculate the number of organisms in the stock. The inoculum was diluted on this basis, and the infectious dose given to the animals was always confirmed by similar culture in HL cells.

4.2 Antibiotic susceptibility studies *in vitro*

MIC and the minimal bactericidal concentration (MBC) of telithromycin and levofloxacin against *C. pneumoniae* K7 were determined in HL cell cultures. Cells grown on 24-well plates were inoculated with equal amounts (about 0.5x10³ IFU/well) of chlamydia, and twofold dilutions (between 0.0039 and 2 μg/ml) of the antibiotics were tested in triplicate. The cell culture medium was free of other antibiotics and contained 1 mg/L of cycloheximide. For MIC analysis, infected cells with antibiotic were incubated for 72 hours, fixed and stained with *Chlamydia* genus-specific monoclonal antibody as
described above. MIC was defined as the concentration at which more than 95% of *C. pneumoniae* inclusion formation was inhibited after the first growth cycle. For MBC analysis, the infected and treated cells were transferred on to a new layer of fresh HL cells after the first incubation, the plates were centrifuged, and the cells were incubated for another 72 hours without any antibiotics in the cell culture medium. The concentration at which no inclusions were detected after a passage in antibiotic-free media was defined as MBC.

### 4.3 Animal model and intranasal inoculation

The wild-type mouse strain C57BL/6J is susceptible to both atherogenic changes and *C. pneumoniae* lung infection, as mentioned earlier. It was chosen for these studies since it has been used previously by our group (Erkkilä *et al.* 2004) and to avoid the possible confounding factors of genetic manipulation or atherogenic diets when assessing the effects of infection on the early stages of atherosclerotic development. Female C57BL/6J mice were purchased from M&B A/S, Ry, Denmark, for the telithromycin studies (I, II) and the levofloxacin experiments (unpublished results). The mice for the studies assessing the effects of repeated *C. pneumoniae* inoculations (study III), the effects of flavonoids and octyl gallate on acute infection (study IV) and the effects of acute infection on endothelial function (Study V) were from Harlan, Netherlands. The mice were obtained at the age of six weeks and were allowed to adapt to the facilities for at least two weeks before the inoculations or treatments were started. The mice were kept in standard plastic cages inside ventilated containers (Scantainer, Scanbur A/S, Køge, Denmark). After inoculations, the plastic cages were covered with filtered lids. The diurnal rhythm was 12h light and 12h dark, and temperature and relative humidity were controlled within each container. Water and food were available *ad lib.*, and the mice were fed with normal chow or chow with 0.2% cholesterol supplementation (Altromin, Denmark).

The study protocols for all of the original studies are presented in Table 4. The mice were infected intranasally, and the primary inoculation was given at different ages, depending on the study. The studies I, IV and V assessed the responses only to acute infection, and in the other studies the possible reinfections were always given at four-week intervals. In the chronic infection treatment study with telithromycin (study II) and in the levofloxacin experiments, the diet supplemented with 0.2% of cholesterol was started at the age of six weeks and was given to the mice throughout the study. In study III assessing the effects of repeated inoculations, all the other groups received a cholesterol-supplemented diet from the beginning of the study, except the group IV. For this group, the cholesterol-supplemented diet was started at the age of 18 weeks. The mice in the studies I, IV and V were given a normal chow diet. For intranasal inoculation, the mice were anaesthetised with methoxyflurane inhalation analgesic (Medical Developments Australia; Metofane®, Schering-Plough, USA). An inoculum dose of around 1x10⁶ IFU/mouse has been shown to induce severe lung inflammation and systemic spread of chlamydia in these mice (Penttilä *et al.* 1998a, Erkkilä *et al.* 2004), yet without serious clinical illness or death of the animals. Each dilution of the stock solution
for the present studies yielded slightly variable inoculum concentrations around 1x10^6 IFU. An inoculum dose in 40 μl of SPG buffer pipetted into the nostrils of the mice was inhaled into the lungs during anaesthesia. To collect samples, mice were euthanased with CO₂, blood was immediately collected by heart puncture, and organs were dissected using a clean set of equipment for each mouse. All procedures involving animals were approved by The Animal Care and Use Committee of National Public Health Institute, Helsinki, Finland.

Table 4. Study protocols of the original articles.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study groups</th>
<th>No of mice per group</th>
<th>No. of inoculations</th>
<th>Age at inoculation</th>
<th>Inoculum dose</th>
<th>Sample times</th>
</tr>
</thead>
<tbody>
<tr>
<td>I – acute infection and telithromycin</td>
<td>25 mg/kg; 5/10 days</td>
<td>10/10</td>
<td>1</td>
<td>8 wks for all</td>
<td>6x10^5</td>
<td>8/13 and 21 days p.i.</td>
</tr>
<tr>
<td>II – Chronic infection and telithromycin</td>
<td>50 mg/kg, 5/10 days; 100 mg/kg, 5/10 days; uninfected control</td>
<td>10/10; 9/10</td>
<td>1</td>
<td>IFU/mouse for all groups</td>
<td>1.05-2.2x10^6</td>
<td>4 and 12 weeks p.i.</td>
</tr>
<tr>
<td>III – repeated inoculations with phenotype compounds</td>
<td>20 mg/kg quercetin; 20 mg/kg octyl gallate; 2 mg/kg luteolin; infected control + placebo; uninfected control</td>
<td>8-10/aortic; 8-9/other; 8</td>
<td>1</td>
<td>8 wks for all</td>
<td>2x10^5</td>
<td>3, 6, 10, 13 weeks p.i.</td>
</tr>
<tr>
<td>IV – treatment experiments</td>
<td>50 mg/kg levofloxacin; 100 mg/kg levofloxacin; 100 mg/kg erythromycin</td>
<td>6-10/aortic; 20/other; 10</td>
<td>3</td>
<td>16 wks for all</td>
<td>2.8x10^5</td>
<td>2 and 12 weeks p.i.</td>
</tr>
</tbody>
</table>

*C. pneumoniae* reinoculations were always given at four-week intervals; *b*age at which the primary inoculation was given; *c*times of sample collection, p.i. (post infection) indicates the time after the last inoculation.
4.4 Treatments

4.4.1 Antibiotic treatments

Antibiotic treatments were always started on the third day after the primary, secondary or third inoculation. The doses and treatment times were based on previously published murine models and advice from the pharmaceutical companies Aventis Pharma and Daiichi. The telithromycin regimen for the treatment of chronic infection (study II) was based on the results of the acute infection trial (study I). Telithromycin (in 0.1% acetic acid) treatments were administrated subcutaneously once daily (I, II) and levofloxacin and erythromycin (in 1% carboxymethylcellulose) orally by gavage once daily. To test the effect of telithromycin on acute lung infection, placebo or three different doses of telithromycin, 25, 50 and 100 mg/kg, were given for five- and ten-day treatment periods (I). The animals were sacrificed and samples were taken after 5 days (day 8 post-inoculation, p.i.) and ten days (day 13 p.i.) of treatment and at 21 days p.i.

In the study assessing the effect on the development of chronic infection, 75 mg/kg of telithromycin was given for five or ten days (II). Two groups were inoculated twice with chlamydia, and one of these was treated for ten days after both inoculations to study the effects of early treatment. Another group was treated for ten days after the last inoculation (delayed treatment). The placebo group was given diluent after both inoculations. The effects of delayed treatment were also studied in the fourth group, which was inoculated three times and treated only after the last inoculation for five days. The other placebo group was inoculated three times and received diluent after the last inoculation for five days. Samples were taken two and 12 weeks after the last inoculation.

Levofloxacin and erythromycin were tested to assess the effects of these treatments after the establishment of a chronic infection with three repeated inoculations. Levofloxacin (50 and 100 mg/kg) and erythromycin (100 mg/kg) treatments were given for seven days after the third inoculation, and samples were taken two and 12 weeks after the third inoculation. A placebo group infected with chlamydia and given diluent instead of antibiotics and an infection control group mock-inoculated with SPG were included in the study.

4.4.2 Flavonoid treatments

Two separate studies on flavonoid treatments were performed. Treatments with 20 mg/kg of quercetin (Quercetin dihydrate, Carl Roth, Karlsruhe, Germany), 2 mg/kg of luteolin (Extrasynthese, Genay, France), 20 mg/kg of octyl gallate (Fluka Chemie, Buchs, Switzerland) and diluent (1% DMSO) for the control groups were started and administered once daily intraperitoneally. The treatment regimens were chosen after a preliminary study with two different doses (20 and 2 mg/kg) of each compound (unpublished data) as well as based on previously published animal models and MIC values obtained from in vitro studies (Alvesalo et al. in press). On the fourth day of treatment, the mice were inoculated intranasally with chlamydia in both studies.
Flavonoid treatment on the day of inoculation was given to the mice 4 to 5 hours prior to infection. The treatments were given similarly once daily after the inoculation for ten days in the first study. Samples were taken on the days 3, 6, 10, 13 and 20 p.i. A similar protocol was used in the second study, but the mice were treated only with quercetin and luteolin for four days prior to inoculation and until four days p.i., when the samples were collected. Lung tissue specimens from these mice were collected for the analysis of NOS and cyclooxygenase (COX) mRNA expression, and the aortas were collected for the analyses of endothelial function presented in study V.

4.4.3 Cortisone treatments

Mice inoculated three times and treated after the third inoculation with telithromycin, erythromycin or levofloxacin and the corresponding placebo groups were given cortisone acetate treatment (125mg/kg) to reactivate possible persistent infection in the lungs. Treatments were started either two (levofloxacin, erythromycin) or four (telithromycin) weeks after the third inoculation, and five doses of cortisone were given subcutaneously every other day. Samples from these mice were taken one day after the last cortisone dose.

4.5 Chlamydial culture from lung tissue samples

Lobes from the right lung were mechanically homogenized in 2 ml of SPG buffer. The tissue suspensions were centrifuged at low speed to remove debris, and the supernatant was collected and frozen at -70°C. All analyses were performed in duplicate. HL cells inoculated with 3, 30 and 300 μl of the homogenate were centrifuged, incubated, fixed and stained as described above (studies I, III-V). In studies with repeated inoculations followed by one of the antibiotic treatments or cortisone treatment (II, levofloxacin experiments), the cells were centrifuged again after the first incubation period, new culture medium was added, and the cultures were grown for another 72 h. Finally, the cells were washed, fixed and stained as described before and analysed for the presence of chlamydial inclusions.

4.6 Serological analyses

C. pneumoniae total IgG antibodies were measured with the MIF test, in which whole EBs of the K7 isolate as antigen were formalin-fixed on a special object glass and incubated for 90 min at +37°C with twofold dilutions of the sample serum. IgG antibodies bound to the antigen were detected using FITC-conjugated anti-mouse IgG (F(ab')2 fragments, Serotec). In addition, total C. pneumoniae IgG and immunoglobulin subtypes IgG1 and IgG2c were measured in study III using EB-coated enzymeimmunoassay (EIA) plates (Coated Microstrips®, Labsystems). Sample dilutions
were 1:100 for total IgG and 1:50 for IgG subtypes. Samples were incubated for 90 min at +37°C, and bound antibodies were detected by alkaline phosphatase-conjugated goat anti-mouse IgG, in dilution 1:3000 (Sigma) and goat anti-mouse IgG1 and IgG2c, in dilutions 1:4000 (SouthernBiotech), with Phosphatase Substrate (Sigma).

Mouse Hsp60 antibodies were measured by the EIA method (study III and levofloxacin experiments) using 96-well plates coated with 5 μg/ml recombinant mouse Hsp60 protein (StressGen Biotechnologies Corp.). Serum samples were diluted 1:20 and incubated on the plates for two hours. Alkaline phosphatase-conjugated anti-mouse IgG and Phosphatase Substrate, as above, were used to detect the specific antibodies. Chlamydial Hsp60 IgG1 and IgG2c (III) were measured with the same method, except that the plates were coated with the chHsp60 protein produced at National Public Health Institute, Helsinki, Finland (Airaksinen 2003) and 1:40 dilutions of the serum samples were used. For the final results, the photometer absorbance values from each EIA analysis were multiplied by the sample dilution to obtain the EIA units.

4.7 DNA detection

After centrifugation of the lung tissue homogenate for chlamydial culture, the remaining lung tissue debris was stored at −70°C for PCR analyses of chlamydial DNA. 50 mg of tissue debris was lysed with proteinase K (Qiagen) in lysis buffer after cutting the tissue into smaller pieces with disposable scalpels (I, II, levofloxacin experiments) or after homogenisation of the tissue with Lysing Matrix D tubes (Q-BIOgene) using the FP 120 FastPrep Cell Disruptor (Savant Instruments, Inc.) (III-V). After proteinase incubation at 56°C overnight, DNA was purified using a commercially available QIAamp tissue kit (Qiagen) according to the manufacturer’s instructions. The purified DNA was kept frozen at -20°C.

In addition, aortic tissue samples from the mice analysed for endothelial function in study V were homogenised to extract proteins for Western blot analysis of COX and NOS enzymes (results not shown). The supernatant separated by centrifugation after homogenisation was analysed with the nested PCR method for the presence of chlamydia.

4.7.1 Conventional PCR with time-resolved fluorescence (TRF) detection (I, II)

Primers used for DNA amplification were modified from those originally published by (Holland et al. 1990a) and (Rasmussen et al. 1992) and synthesized at the Institute of Biotechnology, Helsinki, Finland. The biotinylated HB2 (forward) and HB1 (reverse) primers producing a 135 bp sequence and Europium-labelled hybridisation probe were derived from the C. pneumoniae ompA gene (Table 5). PCR was performed in a mixture of 400 μM of each deoxynucleoside triphosphate, 4.0 mM MgCl2, 50 mM Tris-HCl (pH 8), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 1% Triton X-100, 1.0 U
Tag polymerase (PromegaTag), 50 pmol of each primer and 10 μl of isolated DNA sample. Total reaction volume was 50 μl. After 5 minutes of denaturation at 94°C, the samples were subjected to 50 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The presence of C. pneumoniae-specific PCR product was analysed by TRF-based hybridisation assay using the Europium-labelled hybridisation probe. A 10 μl volume of amplified PCR product was transferred in triplicate to streptavidin-coated microtitration wells together with 60 μl of Assay buffer (Perkin Elmer Life Sciences Wallac Oy), and the sample was allowed to attach to the wells at room temperature. After 30 min incubation, the wells were washed three times with DELFIA wash solution (Perkin Elmer Life Sciences Wallac Oy), and DNA was denatured in 50 mM NaOH. After washing, an excessive amount of hybridisation probe (2 ng/100 μl) was added to the wells and incubated overnight at +37°C. The hybridisation reaction was stopped by washing ten times. Enhancement solution was added and, after 30 minutes of incubation by shaking, the signal was detected using a Delphia 1234 time-resolved fluorometer (Perkin Elmer Life Sciences Wallac Oy). The detection limit for the PCR assay was 0.8 genome equivalents. All positive samples in the first run were assayed twice and considered positive only if the result was the same from both runs.

4.7.2 Real-time 16S rRNA PCR (IV)

Quantitative real-time analysis of chlamydial 16S rRNA was done with the Roche LightCycler system using a FastStart DNA Master Hybridisation Probes kit. Primers CpnA (forward) and CpnB (reverse), previously published by (Gaydos et al. 1992b), and specific hybridisation probes CP16FL and CP16LF published by (Reischl et al. 2003), were obtained from TIB Molbiol (Germany). The sequences for the primers and probes are shown in Table 5. The LightCycler PCR reaction mixture contained 3mM MgCl₂, 0.5 μM of each primer, 0.2 μM of each probe, 2 μl of 10x FastStart DNA Master Hybridisation Probes mixture, and 8 μl of template in a final volume of 20 μl. In the amplification protocol, preincubation for 10 minutes at 95°C was followed by 50 cycles with 10 s at 95°C, 10 s at 50°C and 20 s at 72°C. For quantification, C. pneumoniae EBs cultured in HL cells were purified by Urografin density gradient ultracentrifugation, and DNA was extracted by a QIAamp tissue kit and analysed using a spectrophotometer. The numbers of C. pneumoniae genomes were calculated using the known molecular weight of one genome, and the stock was diluted to obtain standards from 1 to 10⁷ genomes/μl. Using these as a standard curve, the quantification of the samples was calculated by the second derivative maximum method of the LightCycler Data Analysis software (version 3.5.28). Samples were considered C. pneumoniae-positive if at least a 0.3 x rise of fluorescence (F2/F1) compared to background fluorescence was seen.

4.7.3 Combined conventional and real-time nested PCR (III, V)

Nested PCR assay combining conventional and real-time PCR methods was used to detect the presence of C. pneumoniae DNA in lung tissue samples in study III. The first
round of nested PCR was conventional, using outer primer pair pstfor (forward) and pstrev (reverse) (Table 5), which amplify a product of 192 bp of the *C. pneumoniae* *pst* fragment. This primer pair was designed using Primer 3, and their specificity was tested using BLAST for short, nearly exact matches. To prevent carryover contamination, heat-labile uracil-DNA-glycosylase (UNG) enzyme was added, and dTTP was replaced by dUTP in the first step of our PCR. Negative controls and tenfold dilution series of *C. pneumoniae* DNA standard (800, 80 and 8 copies) were included in each run. The reaction volume was 50 μl with 3.0 mM MgCl₂, 5 μl of 10x PCR buffer (Promega), 0.4 μM of both primers, 200 μM of dATP, dGTP and dCTP (Promega) and 600 μM of dUTP (Roche Diagnostics GmbH), 2 U of Taq polymerase (Promega) and 1 U of UNG enzyme (Roche Diagnostics GmbH). The PCR program included initial incubation for 10 min at 20 °C for the UNG enzyme to cut possible contaminating PCR products and 2 min at 95 °C for denaturation of the enzyme. This was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 30 s. The PCR products were diluted 1:10 and 1:100, and 8 μl of these dilutions were used for the second round of real-time PCR done with LightCycler. The second-round PCR was adapted from an article by (Ciervo et al. 2003). In this protocol, the inner primers ln-1 (forward) and ln-2 (reverse), originally from (Maass et al. 1997), amplify a 128 bp product of the *pst* fragment, and the product is recognized specifically by the probes CP1-Flu and CP1-LC (Table 5). All negative controls, first-round standards and a new dilution series of *C. pneumoniae* DNA standard (8 to 8x10⁴ copies) were included in each run. The reaction volume was 20 μl with 4.0 mM MgCl₂, 0.4 μM of each primer, 0.3 μM of the probe CP1-LC, 0.2 μM of the probe CP1-Flu and 2 μl of 10 x FastStart DNA Master Hybridisation Probes mixture. The PCR program consisted of preincubation at 95°C for 10 min, 55 cycles at 95°C for 10 s, 57°C for 8 s and 72°C for 10 s. A standard curve was generated from a series of dilutions of *C. pneumoniae* DNA as described above, and the results were analysed using the second derivative maximum method of the LightCycler Software (version 3.5.28). Samples were considered *C. pneumoniae* positive if at least a 0.3x rise of fluorescence (F2/F1) compared to background fluorescence was seen.
Table 5. Primers and probes used in the PCR analyses to detect C. pneumoniae DNA.

<table>
<thead>
<tr>
<th>Method and target</th>
<th>Primer/probe</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional PCR</td>
<td>HB2</td>
<td>5'-biotin-CCTGTAGGGAACCTCTGATC-3'</td>
<td>study II</td>
</tr>
<tr>
<td>omp1 gene</td>
<td>HB1</td>
<td>5'-ATAGTCTCCGTTAAAATCCAGCAG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eu-probe</td>
<td>5'-Eu&lt;sup&gt;-&lt;/sup&gt;-CCATATTGACCATCAATTA-3'</td>
<td></td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>CpnA</td>
<td>5'-TGACAACCTGTAGAAATACACG-3'</td>
<td>Gaydos et al. 1992b</td>
</tr>
<tr>
<td>Gene for 16S rRNA</td>
<td>CpnB</td>
<td>5'-CGCCTCTCTCTCTAAAAAT-3'</td>
<td>al. 1992b</td>
</tr>
<tr>
<td></td>
<td>CP16FL</td>
<td>5'-GTAGCAAGATCGTAGGAGAACAA-(FL)&lt;sup&gt;-&lt;/sup&gt;3'</td>
<td>Reischl et al. 2003</td>
</tr>
<tr>
<td></td>
<td>CP16LC</td>
<td>5'-&lt;Red 640&gt;-TCCTAAAAGCTAGCCCCAGTTCC-(Ph)&lt;sup&gt;-&lt;/sup&gt;3'</td>
<td>al. 2003</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>outer</td>
<td>5'-AAACGGGATTACAAACGCTA-3'</td>
<td>study III</td>
</tr>
<tr>
<td></td>
<td>inner (real-time)</td>
<td>5'-GGGAACGATTTTTGAAACAA-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ln-1</td>
<td>5'-AGTTGAGCATATCTGTGAGG-3'</td>
<td>Maass et al. 1997</td>
</tr>
<tr>
<td></td>
<td>ln-2</td>
<td>5'-TTATTTTGGTGCCTGAGCAG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP1-Flu</td>
<td>5'-CTCTTGAAGCAACGTCAGAC-(FL)-3'</td>
<td>Ciervo et al. 2003</td>
</tr>
<tr>
<td></td>
<td>CP1-LC</td>
<td>5'-&lt;Red 640&gt;-TAACCTTGCGGAATGACCCA-(Ph)-3'</td>
<td>al. 2003</td>
</tr>
</tbody>
</table>

*Europium label, *fluorescein, *phosphate

4.8 RNA detection

About 10-30 mg of tissue from the right lung was placed in RNA stabilization solution (RNA<sub>later</sub>™, Qiagen) and stored frozen at −20°C for RNA analysis. RNA was extracted using High Pure RNA Tissue Kit (Roche). Proteinase K incubation at +56°C for one hour, not included in the kit procedure, was done after homogenisation of the tissue by FastPrep Cell Disruptor, and the extracted RNA was eluted 2 times with 50 μl of elution buffer. Otherwise, the extraction was done as advised by the manufacturer. The procedure included a DNase incubation step to disrupt DNA.

4.8.1 Chlamydial groEL expression (III)

The PCR primers for the analysis of C. pneumoniae Hsp60 gene groEL expression were designed with the Vector NTI (InforMax Inc.) program, and their specificity was tested using BLAST for short, nearly exact matches. The primers were ordered from TibMolbiol (Germany). The sequences of the outer primers GroEl-1 (forward) and GroEl-2 (reverse) for the nested PCR and the inner primers GroEl-3 (forward) and GroEl-4 (reverse) are shown in Table 6. To obtain cDNA, about 1 μg of total RNA was transcribed using 10 units of Transcriptor Reverse Transcriptase enzyme (Roche) with 1 mM of each dNTPs and 1.5 μM of specific GroEl-2 reverse primer in a final volume of 20μl. The reverse transcriptase (RT) reaction was done at 55°C for 30 minutes, after which the enzyme was inactivated at 85°C for 5 minutes. 5 μl of cDNA was taken for conventional PCR amplification using 0.3 μM of the outer primers GroEl-1 and GroEl-2 with 0.2 mM of
each dNTPs (Promega), 2 mM MgCl₂ and 1.5 units of Taq-polymerase (Promega) in a final volume of 50 µl. The amplification program included preincubation at +94°C for 3 min, 30 cycles of 30s at 94°C, 30 s at 56°C and 30 s at 72°C and a final extension of 5 min at 72°C. Positive standards of 2, 20 and 200 C. pneumoniae genome copies were included in the outer, conventional PCR. All products from conventional PCR were diluted 1:100 and amplified by LightCycler together with C. pneumoniae standards (tenfold dilutions from 2 to 2x10⁷ copies). The LightCycler reaction mixture included 2 µl of diluted template from conventional PCR, 0.5 µM of the primers GroEl-3 and GroEl-4, 3 mM MgCl₂ and 2 µl of the LightCycler FastStart DNA Master CYBR Green I reaction mix. The amplification program consisted of 10 min pre-incubation at 95°C and 40 cycles at 95°C for 10 s, 61°C for 5 s and 72°C for 10 s. After amplification, melting curve analysis between the target temperatures from 70°C to 95°C was performed to verify the products. Real-time amplification was analysed using the LightCycler Data Analysis program’s Second derivative maximum method (LightCycler Software version 3.5.28). Total RNA from a culture-positive mouse lung sample collected 4 days after intranasal inoculation and stored in RNA stabilization buffer was extracted and analysed among the other samples as a positive control within each run. The criteria used for a positive result were: 1) 2x increase of CYBR Green fluorescence over the background fluorescence level, 2) crossing-point cycle, i.e. the cycle number at which fluorescence started to increase from the baseline, to appear earlier than the crossing-point of the lowest standard on the LightCycler PCR and 3) the melting point from the melting curve analysis to be within the range detected in the standards and control (from 81.5°C to 81.9°C).

4.8.2 Mouse iNOS, eNOS, COX-1 and COX-2 expression (IV, V)

The RT reaction was performed with the Transcriptor Reverse Transcriptase enzyme for 30 min at 55°C using random pd(N)₆ primers (Roche) according to the manufacturer’s recommendations. LightCycler real-time analysis for cDNA was performed by the FastStart DNA Master SYBR Green I kit (Roche) with specific primers obtained from the inducible NOS (iNOS) (Phillipson et al. 2003) and the endothelial NOS (eNOS) (Laufs et al. 2002), COX-1 and COX-2 (Tanigawa et al. 2004) mRNA sequences (Table 6). The primers were ordered from TibMolbiol (Germany). The PCR reaction mixtures with a final volume of 20 µl consisted of 2.5 mM MgCl₂ for iNOS, eNOS and COX-2 and 3 mM for COX-1, 0.5 mM of each primer, 2 µl of FastStart DNA Master CYBR Green I mixture and 2 µl of cDNA template. After pre-incubation at 95°C, the amplification cycles for the different genes were: iNOS 95°C/15s, 61°C/10s, 72°C/10s; eNOS 95°C/15s, 61°C/10s, 72°C/15s; COX-1 95°C/10s, 60°C/10s, 72°C/5s; COX-2 95°C/15s, 61°C/10s, 72°C/9s. A melting curve analysis from 70°C to 95°C was performed to confirm the amplification of a specific product. Specific standards for each gene were produced by purification of the correct PCR product from agarose gel, amplification of this product with LightCycler and concentration of the amplified products by ethanol precipitation. The amount of specific sequence in each stock was determined and diluted to obtain
standards of 1 to $10^4$ genomes/μl. Quantification was done by using a standard curve as described in C. pneumoniae 16S rRNA LightCycler analysis.

The expression of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured from the cDNA samples by LightCycler using FastStart DNA Master SYBR Green I kit (Roche). The method was obtained from (Simpson et al. 2000). Briefly, the PCR mixture contained 4 mM MgCl₂, 0.5 μM of each primer, 2 μl of FastStart DNA Master SYBR Green I mixture and 2 μl of 1:10 diluted cDNA template in a final volume of 20 μl. Preincubation at 95°C for ten minutes was followed by 45 cycles at 95°C for 10 s, 57°C for 5 s and 72°C for 8 s. As above, the crossing-point value represents the PCR cycle at which the increase in the SYBR Green fluorescence signal above the baseline was detected and indicates the level of template concentration in the sample. Crossing-points for GAPDH varied significantly in the different study groups (uninfected control: 25.4 (0.99); infected control: 24.7 (0.99); quercetin: 25.1 (0.28); luteolin 26.3 (0.48); averages (SD)). To some extent, infection and especially luteolin treatment affected the mRNA levels of GAPDH, and it was therefore not suitable to be used in the relative quantification of NOS and COX levels. However, a significant negative correlation ($r=-0.524$, $p=0.006$) was detected between the crossing-point values for GAPDH and the weight of the tissue taken for RNA extraction. The NOS and COX cDNA absolute quantification calculated by the LightCycler software was therefore adjusted for tissue weight. The results are presented as per 25 mg of lung tissue.

Table 6. Primers used in the RT-PCR analyses to detect specific gene expression levels.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydial groEL gene</td>
<td>outer (conventional)</td>
<td>study III</td>
</tr>
<tr>
<td></td>
<td>GroEl-1: 5’-AAG TAG CAG AAT CTG GAC GCC C-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GroEl-2: 5’-CGG ACA CCA CCG GAG AGT TTA-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inner (real-time)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GroEl-3: 5’-TCT GTG CAG TGA AAG CTC GTG G-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GroEl-4: 5’-CGC ATC GAG CTT GGA TAT CAG G-3’</td>
<td></td>
</tr>
<tr>
<td>Mouse iNOSα</td>
<td>Forward: 5’-CAGCTGGGCTGTACAAACCT-3’</td>
<td>Phillipson et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CATTGGAAAGTGAGGCTTTCG-3’</td>
<td></td>
</tr>
<tr>
<td>Mouse eNOSβ</td>
<td>Forward: 5’-TTCCGGCTGCCACCTGATCTAA-3’</td>
<td>Laufs et al. 2000</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-AACATATGTCTCTTGGCTAAGGCA-3’</td>
<td></td>
</tr>
<tr>
<td>Mouse COX-1γ</td>
<td>Forward: 5’-CATCAAGGAGTCCCCGAGAGAT-3’</td>
<td>Tanigawa et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TAAGGCTTCAAGCAACCCCTC-3’</td>
<td></td>
</tr>
<tr>
<td>Mouse COX-2</td>
<td>Forward: 5’-AAGCCCTCCTACAGTGACATCGA-3’</td>
<td>Tanigawa et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-ATGGTCTCCCCCAAGATAGCAT-3’</td>
<td></td>
</tr>
<tr>
<td>Mouse GAPDHδ</td>
<td>Forward: 5’-AAGCGACCCCTTCCATTGAC-3’</td>
<td>Simpson et al. 2000</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TCCACGACATCTCAGCAC-3’</td>
<td></td>
</tr>
</tbody>
</table>

αinducible nitric oxide synthase, βendothelial nitric oxide synthase, γcyclooxygenase, δglyceraldehyde-3-phosphate dehydrogenase
4.9 Lung and aortic tissue histopathology

The left lungs from mice in each study were removed and fixed in 10% buffered formalin. The fixed tissue was cut into three transversal sections, and the pieces were embedded in paraffin. 4 μm sections including a section from each of the three pieces were attached on to an object glass and stained with haematoxylin-eosin (HE). The severity of bronchointerstitial pneumonia, consisting mainly of perivascular and peribronchial lymphocyte and plasma cell infiltration, was assessed and evaluated on a scale from 0 to 4. Occasional neutrophilic and eosinophilic granulocytes were also present, especially during the first few days after primary inoculation. No inflammatory reactions were visible in grade 0, cell infiltration was mild in grade 1, and the changes were moderate in grade 2, marked in grade 3 and severe in grade 4. Grade 4 also included patchy consolidation of lung tissue with alveolar macrophages and plasma cells.

After the lungs had been dissected in study II, the heart and the adjoining aorta were perfused with 1 ml of PBS, removed and fixed in 10% buffered formalin. The aorta and the heart were cut apart from each other, and the sites of the three carotid arteries branching off were separately embedded in paraffin. Transversal 4μm sections were cut from the branching sites of the aortic arch and stained with HE. From five mice per group, the upper half of the heart was also embedded in paraffin, and 4 μm sections from the area of the aortic sinus valve cups (as described below) were collected and stained with HE. All sections were evaluated histopathologically for lesions and inflammation.

4.10 Aortic sinus lipid analyses (II, III, levofloxacin experiments)

To analyse the subendothelial lipid accumulation in the aortic sinus of a mouse, the heart was perfused with 1 ml of PBS, removed and fixed in 10% buffered formalin. The upper half of the formalin-fixed heart was embedded in gelatine and frozen. 5 μm sections were cut with a cryostat microtome from the part of the aortic sinus where the valve cusps and valves were starting to form to where these structures were clearly present. The method of collecting samples and sections has been described by (Paigen et al. 1987). Every other section cut was placed on a specific object slide coated with gelatine, totalling an average of 24 to 30 sections per mouse, and stained with Oil-Red-O. Of these, every third collected section was analysed and the areas with lipid accumulation from six consecutive sections per mouse from the same site were quantified with computer-assisted image analysis. In some mice, very large lesion areas were detected in two or three of the six analysed sections but in none of the rest, whereas in some other animals, moderate-sized lesions were present throughout the six studied sections. Averaging of the lesion areas from sections with such variation between the mice might have given a wrong impression by neglecting either the diameter in the first type of cases described above or the “length” in the case of long but thin lesions. For these reasons, a sum of the lesion areas from six sections was calculated for each mouse, and these values were further analysed.
4.11 Aortic endothelial relaxation studies (V)

To collect aortas for endothelial relaxation studies, the heart and the aorta were perfused with 2 ml of Krebs physiologic salt solution. The aortas were dissected and cleared of surrounding fat, and the descending thoracic and abdominal aorta was divided into 2 sections. The upper section (thoracic aorta) was cut into rings (about 3 mm), which were mounted in an organ bath (EMKA 2000, France) for isometric measurements. The endothelium-intact rings of arteries were placed between stainless steel hooks and mounted in an organ bath chamber. The rings were equilibrated for 60 minutes under a resting tension of 1.2 g in oxygenated (96% O₂ /4% CO₂) Krebs solution (pH 7.4, 37°C). The force of contraction was measured with an isometric force-displacement transducer using a computerized system (EMKA 2000, France). The presence of intact endothelium in the vascular preparation was confirmed by observing the relaxation response to 1 μM methacholine (MC) (Acetyl-B-Methacholine) in rings precontracted with 0.1 μM noradrenaline (NA) (bitartrate salt). To measure the endothelial function, the aortic rings were relaxed with increasing concentrations of MC after noradrenaline contraction. In addition, the effects of the NOS enzyme inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) and the non-selective COX inhibitor, diclofenac (diclofenac sodium salt) on arterial relaxation were measured by incubating the aortic rings with these solutions in organ baths prior to relaxation assays. Noradrenaline, methacholine, L-NAME and diclofenac were from Sigma Chemical Co., St Louis, MO, USA.

4.12 Statistics

Non-parametric Mann-Whitney U-test was used to compare the results of *C. pneumoniae* culture, when appropriate, antibody levels (I, III, IV, levofloxacin experiments), Oil-Red-O-stained lipid accumulation areas (II, III, levofloxacin experiments) and quantitative DNA analyses as well as quantitative analyses of NOS and COX expression (IV, V) between the groups. Spearman’s correlation was used to evaluate the correlation between the different antibody subtypes (III) and the correlation between the GAPDH crossing-point values from the LightCycler analysis and the weights of the mice (IV, V). The percentages of the different histopathology grades between the groups were compared using Fisher’s exact test (I, levofloxacin experiments). PCR positivity percentages in the different lung histopathology grades were compared using Pearson’s chi-square test or Fisher’s exact test, as appropriate, and changes in lung inflammation in the different treatment groups were evaluated by the Chi-square test for trend (II). The endothelial relaxation responses of the groups were compared using 2-way ANOVA for repeated measures (V). These statistics were calculated using SPSS version 10.0.5 (I-III), 11.0.1 (levofloxacin experiments) and 11.5.1 (IV, V). Non-parametric test for trend across ordered groups was used to compare the lung histopathology findings to antibody levels (Stata 5.0; Stata corporation, Inc., College Station, Texas) (III).
5 Results

5.1 Clinical observations

*C. pneumoniae* inoculations led to a self-restricting lung infection and histopathological findings with no signs of clinical illness. Both the lung inflammation responses and the absence of clinical symptoms corresponded to the findings described in the previous animal models with similar inoculum doses, as pointed out earlier in chapter 2.3. In study III, which assesses the effects of multiple inoculations, one animal developed severe pneumonia after repeated inoculations in group I and was euthanased during the study. A slight delay in weight gain was seen in some of the mice after primary inoculation, but no statistically significant differences were seen in body weights in the whole study groups. As shown in study III, the 0.2% cholesterol-supplemented diet did not affect the serum total cholesterol or triglyceride levels of the mice, which remained normocholesterolemic.

In the telithromycin treatment of chronic infection (study II) with subcutaneous treatment injections, the mice inoculated three times had skin irritation and developed sores at the injection sites on their necks for an unknown reason. This was detected mostly in the telithromycin-treated groups, but to some extent also in the placebo groups. For this reason, the treatment in these groups had to be terminated after five days, after which the sores healed rapidly. Consequently, the treatment time after the last inoculation was only five days for these mice (group 4) compared to ten days in the twice-inoculated mice (groups 1 and 2).

5.2 Effects of *C. pneumoniae* infections

5.2.1 Chlamydia culture from lung tissue

After an acute primary infection, 100% of the infected, untreated mice were still culture-positive around two weeks p.i. (I, III), after which culture positivity decreased (I). As
detected in the levofloxacin experiments and in study III with several repeated inoculations, only about 10% of the infected, untreated mice inoculated three times were found positive by culture two weeks after the last inoculation. None of the levofloxacin or erythromycin-treated mice with three inoculations were found positive at this time point. At 4 weeks after repeated (three or more) inoculations, no positive findings were found in any of the study groups (II, III).

Cortisone acetate treatment given two weeks after the third inoculation was not able to reactivate culture positivity in any of the mice in the levofloxacin experiments or in study II even in the case of untreated mice (unpublished data).

5.2.2 Chlamydial DNA

Three different PCR methods were used in these studies to measure chlamydial DNA in tissues. A TRF-based detection method after conventional PCR was used in the telithromycin studies (I and II) to detect the *C. pneumoniae* omp1 gene. The method was able to semi-quantitatively analyse chlamydial DNA in the extracted samples, and it was able to detect small amounts of chlamydial DNA present in the samples from chronically infected mice. Yet, the results were not repeatable in some of the samples, and the method was time-consuming and laborious. Real-time detection of the gene for *C. pneumoniae* 16S rRNA by the LightCycler system, used in the flavonoid study (IV), enables absolute quantitation of the genome copy numbers and is fast to perform. This method, however, was not useful in the analysis of chronic infections. The PCR reaction volume in this system is 20 µl, allowing a maximum of 8 µl of template in the PCR mixture. In addition, the presence of inhibitors of the PCR reaction was detected in the DNA samples extracted from the mice inoculated repeatedly. Although the real-time method for 16S rRNA is sensitive and accurate in measuring the samples collected after acute infection, the small template volume combined with the strong inhibition made the method not sensitive enough in analysing samples from chronically infected mice. To avoid the effects of inhibition, a nested method using conventional PCR for amplification and real-time PCR for both amplification and detection of the product was developed in our laboratory. This method was used in the studies III and V and was able to detect the presence of small amounts of chlamydial DNA in the samples from chronically infected mice.

Despite the negative isolation results in multiple inoculated mice, chlamydial DNA was found to persist in a notable proportion of the animals inoculated repeatedly. In study III, which assessed the effects of repeated inoculations, 60% of the mice that were inoculated three times at the ages of 8, 12 and 16 weeks were still positive by PCR analysis at 32 weeks (Fig. 2B). Among the infected and placebo-treated mice in the telithromycin study with treatment for the chronic infection (study II), persistence of chlamydial DNA was detected in 35% of twice and 59% of three times inoculated mice 12 weeks after the last inoculation, at the age of 24 weeks (Fig. 2A). A decrease in the presence of chlamydial DNA was detected in the group inoculated twice but not in that inoculated three times.
Fig. 2. Percentages of mice per study group with chlamydial DNA present in their lung tissue samples, as measured by the PCR method. A, telithromycin study II, groups: 1) 2 inoculations, 2 treatments; 2) 2 inoculations, 1 treatment; 3) 2 inoculations, no treatment; 4) 3 inoculations, 1 treatment; 5) 3 inoculations, no treatment. * p=0.001 and ** p<0.001 compared to 4 weeks p.i. B, study III, groups: I) inoculated six times, starting at the age of 8 weeks; II) inoculated three times, starting at the age of 8 weeks; III) inoculated three times, starting at the age of 16 week; IV) inoculated three times, starting at the age of 16 weeks + shorter cholesterol feeding. Samples at 2 weeks p.i. were collected at the age of 18 weeks in group II and at the age of 30 weeks in the other groups. p.i. (post-infection) = after the last inoculation.

60% of the aortic tissue samples of all of the chlamydia-inoculated mice in the study evaluating endothelial function (V) were found to be positive when the tissue supernatants, obtained as described in the methods (4,7), were analysed by the nested PCR method targeting the chlamydial pst fragment. Positivity was not found when the DNA samples were analysed with real-time PCR targeting the gene for 16s rRNA.

5.2.3 Chlamydial RNA

The presence of chlamydial RNA in the lung tissue of the mice with repeated inoculations in study III was analysed by RT-PCR targeting the chlamydial Hsp60 gene and combining the conventional and real-time PCR methods. Only lung tissue samples collected at 32 weeks were studied, and none of these were found positive by the method used.
IgG antibodies against whole chlamydial EB particles started to appear around 8 days p.i. and were detected in all of the intranasally inoculated mice. Mice mock-inoculated with SPG did not develop antibodies against chlamydia in any of the studies. The EIA and MIF methods were compared to measure total IgG levels in study III, and both of the methods detected the presence of antibodies equally well, with the exception that the MIF method was better in measuring the very high IgG antibody levels, up to titre 8192, in some of the repeatedly inoculated mice. However, similar responses but with less variation within the groups were seen in the EIA results.

The autoantibody responses against mouse Hsp60 proteins were measured in study III with repeated inoculations and in the levofloxacin experiments to evaluate the autoimmune response induced by chlamydial inoculations in the mice. Infection increased the levels of mHsp60 IgG antibodies, and statistically significant differences between the groups were detected in study III already at 2 weeks after primary chlamydial inoculation (Fig. 3A).

![Fig. 3. Mouse Hsp60 IgG autoantibody levels](image)

IgG1 and IgG2c subtype antibody levels against both chlamydial whole EB particles and chlamydial Hsp60 protein were also measured in study III. Significantly higher IgG2c antibody levels were detected in both of these analyses, but especially against cHsp60. High IgG2c antibody levels indicate a TH1 type immune response against infection, as detected in the previous animal models as well. A weak IgG1 response, detected as a rise
in the cHsp60 IgG1 levels above the baseline, was measured four weeks after the last inoculation only in the mice inoculated six times with chlamydia. The IgG2c antibody levels against cHsp60 were further found to correlate with the severity of the inflammatory response in lung tissue and, importantly, with the mHsp60 autoantibody levels. Spearman’s correlation coefficient at 32 weeks of age between mHsp60 and cHsp60 IgG2c was 0.49, indicating a moderate correlation between the two (Fig. 4).

![Fig. 4. Spearman’s correlation between chlamydial Hsp60 IgG2c and mouse Hsp60 IgG levels in mice at 32 weeks of age (study III).](image)

### 5.2.5 Lung inflammation

Lung inflammatory responses consisting mainly of polymorphonuclear and mononuclear cell infiltrations in peribronchial and perivascular areas were graded on a scale from 0 to 4. Examples of the grades 0-2 and 4 are shown in Figure 5. The most severe, grade 4 inflammatory responses, including consolidation of lung tissue and lymphocyte and plasma cell infiltrations together with alveolar macrophages and plasma cells covering wide areas of all parts of lung tissue were seen on the days 8 and 13 after the primary inoculation. At two weeks after three repeated inoculations, as shown in study III and in the levofloxacin experiments, the response was already milder, showing occasional peribronchial and perivascular cell infiltrations. This is shown in Figure 6, where the histopathology results from the infected control groups of the different studies are combined. Marked and severe inflammation (grades 3 and 4) was no longer detected at 4 and 12 weeks after the last inoculation. However, at 12 weeks after the third inoculation in study II and in the levofloxacin experiments, 42% and 20% of the mice, respectively, had grade 2 inflammation persisting in the lungs. In the uninfected control group, mild inflammation possibly due to the SPG mock inoculation showing very few inflammatory
cell infiltrates detected sporadically (grade 1) was seen in less than 50% of the mice in each study.

Fig. 5. Examples of lung inflammatory reactions after *C. pneumoniae* inoculation measured on a scale from 0 to 4. A, grade 0, no inflammation. B, grade 1, mild inflammation. C, grade 2, moderate inflammation. D, grade 4, severe inflammation.
Fig. 6. Lung histopathology findings at different time points post-infection (p.i.). The lines represent the averages and standard deviations of the lung inflammation grades of the infected, placebo-treated control groups collected from different studies. The inflammatory responses detected at different time points after the primary inoculation are shown on the left side, and the responses after the second reinoculation are shown on the right side.

5.2.6 NOS/COX expression

Quantifications of iNOS, eNOS, COX-1 and COX-2 cDNA per 25 mg of lung tissue were reported in the flavonoid study (IV) and in the study assessing the endothelial function (V). No significant differences in the expression levels of iNOS, eNOS or COX-1 were detected between the uninfected group and the infected control group four days p.i. in either of the studies. However, the infected control group showed a slight increase in COX-2 expression: the geometric mean (95% CI) of LC quantification per 25 mg of tissue was 261.1 (197.9) copies vs. 155.5 (131.7) copies in the uninfected group (study V), which was close to significant (p=0.074).

5.3 Aortic changes caused by infections

5.3.1 Subendothelial lipid accumulation

Subendothelial lipid and foam cell accumulation in the aortic sinuses of the mice were analysed from Oil-Red-O-stained sections in study III evaluating the effect of repeated inoculations, in the tef lizardmcy study (II) and in the levofloxacin experiments. An example of an analysed lesion is shown in Figure 7. Lipid lesion areas from six aortic sections as described in the methods were quantified, and the sum of the areas from these
six sections was calculated for each mouse. The results from the three studies are shown in Figure 8. The largest lesions in the aortic sinuses were usually present in the area where the valve cusps and valves just started to appear. In the case of samples from a few mice, the cutting of the sinus sections was started too late, and the area of interest was not on the collected sections. These mice were excluded from the final analysis.

Fig. 7. An example of an Oil-Red-O-stained section of the aortic sinus of a mouse inoculated three times with *C. pneumoniae* and treated with telithromycin after the last inoculation. The figures show three consecutive sections of one animal and an enlargement of the large lesion seen in the first section.

In study III with repeated inoculations, the area of a single lesion in the sections collected at the age of 32 weeks varied from 0 to 6288 \( \mu \text{m}^2 \). As shown in Figure 8A, six repeated *C. pneumoniae* inoculations in group I increased the lesion areas significantly when compared to the uninfected mice. Relatively large lesions were detected in individual mice in every study group, including the uninfected controls, but mice with small lesion areas were lacking in group I. The difference between group I (geom. mean of the group 6542 \( \mu \text{m}^2 \)) and the control mice in group V (1376 \( \mu \text{m}^2 \)) was statistically significant (\( p=0.034 \)). The lipid lesion areas in group IV inoculated at an older age with the shortest period of cholesterol feeding were comparable to the control mice and significantly smaller compared to group I (1399 \( \mu \text{m}^2 \), \( p=0.045 \)). The mice inoculated three times did not differ significantly from the control groups. (Fig. 8A).
Fig. 8. Sums of aortic sinus lipid lesion areas of the mice and geometric means of the different study groups. A, study III assessing the effects of repeated inoculations, groups: I) six inoculations every fourth week; II) three inoculations every fourth week, starting at the age of 8 weeks; III) three inoculations every fourth week, starting at the age of 20 weeks; IV) three inoculations every fourth week, starting at the age of 20 weeks, short cholesterol feeding; V) mock-inoculation with SPG; * and **: significant difference to group I (p=0.034 and p=0.045, respectively). B, telithromycin study (study II), groups: 1) 2 inoculations, 2 treatments; 2) 2 inoculations, 1 treatment; 3) 2 inoculations, no treatment; 4) 3 inoculations, 1 treatment; 5) 3 inoculations, no treatment. * Significantly lower compared to group 2 (p=0.033). C, levofloxacin experiments, groups: 1) levofloxacin 50 mg/kg; 2) levofloxacin 100 mg/kg; 3) erythromycin 100 mg/kg; 4) placebo-treated; 5) mock-inoculated with SPG.

5.3.2 Endothelial function

The effects of chlamydial inoculations on aortic endothelial function are shown in Figure 9. The aortas from all study groups relaxed similarly in response to increasing MC
concentrations when no pretreatments before MC relaxation were used (Fig. 9A). Only an insignificant decrease of 5 to 10%, depending on the MC concentration, was seen in the aortas from the infected control mice compared to the uninfected group. In the uninfected group, the MC-induced relaxation was mainly NO-dependent, as shown by the inhibitory effect of L-NAME (Fig. 9B) and the lack of a similar effect of diclofenac (Fig. 9C). On the other hand, in the presence of infection, the relaxations were dependent on both NOS products (NO) and COX products (prostanoids), since the relaxing effects of MC were antagonized by both L-NAME and diclofenac (Fig. 9B-C) and completely abolished by their combination (Fig. 9D, p=0.031). The difference between the uninfected and infected control groups was significant only after diclofenac pretreatment (p=0.001; relaxation percentages with the highest MC concentration about 90% vs. 60%, respectively, Fig. 9C). No significant difference was seen after L-NAME pretreatment (p=0.17; relaxation percentages about 45% vs. 20%, respectively, Fig. 9B). The effects of in vivo treatment with quercetin and luteolin during inoculation on the aortic endothelial function were also studied (unpublished results), but the treatments did not modify the ex vivo aortic relaxations either in the absence of pretreatments (Fig. 9A) or when NOS or COX products or both were blocked by pretreatments (Fig. 9B-D).
Fig. 9. Aortic relaxation responses to increasing methacholine concentrations. A, relaxation of the aortic rings without pretreatments. B, relaxation after L-NAME pretreatment. C, relaxation after diclofenac pretreatment. D, relaxation after both L-NAME and diclofenac pretreatments. * p<0.05; ** p<0.01.

5.4 Effects of treatments

5.4.1 Susceptibility of isolate K7 to telithromycin and levofloxacin

Telithromycin MIC for the C. pneumoniae strain K7 was 0.03 mg/l (>95% of the formation of chlamydial inclusions inhibited), while MBC was 0.5 mg/l (no inclusions detected in the second passage). The levofloxacin MIC and MBC values were 0.5 mg/l and 2 mg/l, respectively.
5.4.2 Chlamydia culture and PCR findings

The effects of treatment with telithromycin (study I) and with flavonoids and octyl gallate (study IV) on the presence of viable chlamydia in lung tissue were tested in the course of acute infection by chlamydia culture. 10 days of treatment with telithromycin, especially with the higher doses of 50 and 100 mg/kg, totally abolished the presence of viable bacteria from the lung tissue, whereas occasional positive cultures (one of ten mouse) were found in the group treated with 25 mg/kg at 13 and 21 days p.i. As stated earlier, no positive cultures were achieved after repeated inoculations in the telithromycin study or in the levofloxacin experiments. Treatments with quercetin, luteolin and octyl gallate had diverse effects on the chlamydia culture findings: luteolin showed an attenuating effect on the presence of culturable chlamydia in lung tissue compared to the infected control group, and a significant decrease was detected on day 10 p.i. (p=0.035) whereas quercetin treatment increased the presence of viable chlamydia, significantly on day 6 p.i (p=0.016). Octyl gallate decreased the chlamydia load on day 3 p.i. (p=0.039), but at later time points, an insignificant increase or no differences compared to the controls were seen (Fig. 10).

Treatment of acute infection with telithromycin decreased the presence of chlamydial DNA in the lung tissue, but 10% to 20% of the mice treated with the higher doses (50 and 100 mg/kg) were still found to be PCR-positive three weeks p.i. in the acute infection treatment study (I). As also shown in the chronic infection treatment study (II), complete eradication of DNA positivity was not achieved, but telithromycin treatment given after both of the two inoculations seemed to be most effective (Fig. 2A). Delayed treatment after three repeated inoculations with telithromycin had no effect on the persistence of DNA in the lung tissue. In the flavonoid study (IV), the lowest numbers of chlamydia-
positive mice were found in the luteolin-treated group, but no significant differences in genome numbers between the PCR-positive animals and the controls were detected.

### 5.4.3 Lung inflammation and antibody levels

The inflammatory reaction in the lung tissue after primary inoculation decreased in a dose-dependent manner during telithromycin treatment. On day 13 p.i., the inflammation in the mice treated with 100 mg/kg was significantly (p<0.05) milder than that in the mice treated with 25 mg/kg: 70% of the mice treated with the highest dose had grade 1 or no inflammation present at that time point, whereas all of the mice treated with the lowest dose still had grade 2 or 3 inflammation in lung tissue (study I). Telithromycin treatment also effectively decreased the moderate, grade 2 lung inflammation detected 12 weeks after the last inoculation, but only if the treatment was given after each of the inoculations (study II). Delayed treatment after three inoculations was not able to affect the persistence of the inflammation either in study II with telithromycin or in the levofloxacin experiments. Luteolin was also found to very potently suppress inflammation in both of the studies with flavonoid treatments, as reported in study IV: at all time points, grade 4 inflammation was found only in 6 of the 40 mice treated with luteolin, while 16 of the 40 untreated mice had the most severe inflammation.

Telithromycin was shown to delay the chlamydial IgG antibody response after acute infection in study I, but no significant differences in antibody levels were seen after the treatments in repeatedly infected mice. In the flavonoid study (IV), luteolin treatment was found to decrease the IgG antibody response to chlamydia measured on day 20 p.i. compared to the infected control group. Autoantibodies against mHsp60 were measured in the levofloxacin experiments, and no statistically significant differences were seen between the uninfected and the infected, placebo-treated groups in this study (Fig. 3B). However, treatment after three inoculations with both levofloxacin and erythromycin decreased significantly the presence of mHsp60 autoantibodies at 12 weeks after the last inoculation, erythromycin being the most effective treatment (Fig. 3B).

### 5.4.4 NOS/COX expression

Luteolin treatment decreased significantly the expression of the constitutive enzymes, eNOS and COX-1, compared to the infected control group: the geometric mean (95% CI) of luteolin vs. control in eNOS analysis was 5.3 (17.5) vs. 20.2 (7.7) copies (p= 0.042, study IV) and 1462.2 (714.5) vs. 2734 (1116.6) copies, respectively, in COX-1 analysis (p=0.027, unpublished data). Opposite to the general suppressing effect of luteolin, quercetin significantly increased iNOS expression compared to the infected control: 115.6 (57.5) vs. 65.7 (17.6) copies, respectively (p=0.028, study IV).
5.4.5 Subendothelial lipid accumulation

Aortic sinus sections stained with Oil-Red-O were studied after the telithromycin (study II) and levofloxacin treatments. The lesion areas were analysed at 12 weeks after the third inoculation, at the ages of 24 or 28 weeks in the telithromycin study and at the age of 36 weeks in the levofloxacin experiments. In the telithromycin study, in the mice inoculated twice, the lipid lesion areas were significantly smaller in group 1 (treated twice) compared to group 2 (treated only once) (p=0.033). Compared to the placebo group, the group treated twice showed decreased lipid lesion areas, but this difference was not statistically significant. The mice inoculated three times and treated with telithromycin after the last inoculation showed a trend towards decreased lipid accumulation compared to the placebo-treated groups (at the age of 28 weeks), but no statistically significant differences were detected (Fig. 8B).

No difference was seen between the three times inoculated, placebo-treated group compared to the mock-inoculated control group in the levofloxacin experiments (Fig. 8C), thus indicating again that more than three inoculations are needed to effectively increase lipid accumulation in these mice. In the treatment groups, mice treated with 100 mg/kg of levofloxacin showed decreased lipid accumulation compared to the group treated with 50 mg/kg, but statistical significance was not reached (p=0.12).
6 Discussion

Wild-type C57BL/6J mice were used in the present studies to evaluate the in vivo effects of primary and repeated C. pneumoniae infections on the development of acute and chronic infection and also on the inflammatory responses induced by these infections. We analysed the presence of viable chlamydia and chlamydial nucleic acids and the degree of inflammation in lung tissue and used various serological methods to analyse infection-induced antibody responses. The pro-atherosclerotic changes induced by these infections were evaluated by measuring aortic endothelial function and subintimal lipid accumulation in the aortic sinuses of the mice. Finally, we assessed the effectiveness of antimicrobial treatment with telithromycin, levofloxacin and erythromycin antibiotics and treatment with plant-derived phenolic compounds quercetin, luteolin and octyl gallate on these changes.

6.1 Animal model

Intranasal inoculation with chlamydia led to self-restricting pneumonia and spreading of chlamydia from the lungs to other tissues, as already reported in the early C. pneumoniae infection models (Yang et al. 1993, Kaukoranta-Tolvanen et al. 1993). In general, no clinical symptoms were seen in the mice, and severe pneumonia only developed in some rare cases. These animals were euthanased during the experiments. Infection was followed by a rise in the specific IgG antibody levels against C. pneumoniae around day 8 or 10 p.i. in all of the inoculated mice. Further, large numbers of viable chlamydia particles were cultured from lung tissue as early as day 4 p.i. (study V), and faster clearance of culture positivity was detected after two reinfections compared to the primary infection. Analyses in mice with no anti-infective treatments showed IgG1 type antibodies to be present only in trace amounts, whereas IgG2c antibody levels were high, especially after repeated inoculations. The dominance of subtype IgG2c was expected, since the predominance of the TH1 response in chlamydial murine infection models has been shown previously (Penttilä et al. 1998a, Rottenberg et al. 1999). In the present studies, only the group with six repeated chlamydial inoculations had cHsp60 IgG1
antibody levels above the baseline at 32 weeks of age, four weeks after the last inoculation, suggesting an increased TH2 type response in these mice as well.

Chlamydial Hsp60 is known to be abundant during the whole developmental cycle and highly immunogenic (Peeling & Mabey 1999). In study III, high chHsp60 antibody levels correlated with severe lung inflammatory reactions, and importantly, a similar correlation was also found between mHsp60 IgG antibody levels and lung inflammation grades. Furthermore, there was a positive and statistically significant correlation between the mHsp60 IgG and cHsp60 IgG2c antibody levels in these mice. In agreement with the results published recently (Erkkilä et al. 2004), the mHsp60 IgG levels in chlamydia-infected groups compared to SPG mock-inoculated mice were also higher at every time point. These results strongly support the theory that, during infection when both bacterial and host Hsp60 are overexpressed, bacterial Hsp60 may elicit the production of autoantibodies against self-Hsps and thereby worsen the pathologic consequences in the target tissue.

Normal chow diet supplemented with 0.2% cholesterol was used in the telitromycin study II, in study III with repeated inoculations and in the levofloxacin experiments to induce aortic sinus intimal lipid accumulation. As shown in study III, no statistically significant differences were seen in body weights, total cholesterol or triglyceride levels between the groups, and the mice remained normocholesterolemic despite the small cholesterol supplementation in their diet. Mice carry most of their lipids on HDL (Chapman 1986), and atherosclerotic lesions do not develop spontaneously in these animals. However, the wild-type C57BL/6J mouse strain is prone to develop atherosclerotic changes (Paigen et al. 1985), and these mice are commonly used as a model to study metabolic syndrome (Collins et al. 2004) by feeding them a high-fat and high-cholesterol diet containing cholic acid, i.e. the atherogenic diet. C57BL/6J mice are also susceptible to C. pneumoniae infection (Penttilä et al. 1998b), and intranasal inoculations have been shown to promote advanced atherosclerotic lesions in mice on an atherogenic diet (Blessing et al. 2001). However, both genetic manipulation in knock-out mice and atherogenic diets appear to affect the inflammatory system. Impaired immune responses have been reported in ApoE-deficient mice (Roselaar & Daugherty 1998, Laskowitz et al. 2000), and Laskowitz et al. suggested ApoE to have an important role, besides the lipoprotein metabolism, also in modulating the immune response. The cholesterol component of the atherogenic diet, on the other hand, induces the gene and protein expression of several mediators of acute inflammation, such as serum amyloid A family, and cholate in the diet enhances the production of extracellular matrix proteins, as seen in hepatic fibrosis in response to chronic injury (Vergnes et al. 2003). Several in vivo changes that are part of the atherosclerotic process and due to, for instance, infections by different pathogens can be studied without overt hypercholesterolemia present. To avoid the possible confounding effects caused by genetic or dietary manipulations, normocholesterolemic C57BL/6J mice were used in the present studies, and this model proved to be suitable here.
6.2 Development of chronic infection and persistence of chlamydia

These studies support the view that chronic lung infection persisted in the mice. Lung tissue samples from a large number of mice were positive for chlamydial DNA by PCR in the telithromycin study (II) and in study III several weeks after the last inoculation with chlamydia. Grade 2 inflammation in the lung tissue of the inoculated mice also persisted in the studies II and III and in the levofloxacin experiments. This moderate perivascular and peribronchial infiltration of mononuclear cells was not seen in any of the mock-inoculated control mice. In addition, as shown in the telithromycin study with chronic infection, none of the inoculated, placebo-treated control mice, which were PCR-negative, had inflammatory reactions detected in lung tissue. Previously, it has been shown that alveolar macrophages and peripheral blood mononuclear cells are positive for chlamydial DNA for at least one week after the primary inoculation in C57BL/6J mice, but UV-inactivated chlamydial particles are rapidly cleared from the system (Moazed et al. 1998). This suggests that detected PCR positivity indeed originates from viable bacteria.

Despite the persistence of inflammation and chlamydial DNA in lung tissue, chlamydial mRNA targeting the \textit{C. pneumoniae groEL} gene was not detected in study III. This gene coding \textit{C. pneumoniae} or \textit{C. trachomatis} Hsp60 protein has been shown to be up-regulated in murine alveolar macrophages (Haranaga et al. 2003) and in some (Dean & Powers 2001, Molestina et al. 2002) but not all (Mathews et al. 2001, Belland et al. 2003) previous \textit{in vitro} studies after IFN-\textgamma-induced persistence of infection. Whether this reflects correctly the state of persistence \textit{in vivo} is not clear. However, Gerard et al have previously detected the presence of transcripts of chlamydial Hsp60 in both \textit{C. trachomatis} (Gerard et al. 1998) and \textit{C. pneumoniae} (Gerard et al. 2000) DNA-positive synovial samples. Likely, the negative result obtained in study III was due to the small sample size and possible degradation of chlamydial RNA during the long storage of the samples. Lung tissue samples collected four days after an acute infection for study V and stored similarly for one year had high levels of chlamydial RNA present when measured with the same RT-PCR method. However, in possible chronic infection, the levels of chlamydial mRNA can be assumed to be marginal compared to those seen during acute infection.

Reactivation of the possible chronic infection after repeated inoculations by cortisone acetate did not succeed in study II assessing the effect of telithromycin or in the levofloxacin experiments. Activation of viable chlamydia during a culture-negative stage after the primary inoculation with similar cortisone doses has previously been successful in \textit{C. pneumoniae} and \textit{C. trachomatis} infection models in mice (Yang et al. 1983, Malinverni et al. 1995a, Laitinen et al. 1996). Blocking of the inflammatory reactions restraining chlamydia infection in lungs by cortisone was suggested as an effector mechanism. All these studies, however, detected the reactivation after the primary inoculation, and immunosuppressive treatment was introduced relatively soon after culture negativity had been detected in lung tissue. Yang and co-workers showed in a \textit{C. trachomatis} lung infection model that the effects of cortisone are dose-dependent (Yang et al. 1983). Therefore, to abolish the protective immunity after repeated inoculations, stronger immunosuppression might be needed than that used after the primary
inoculation. The detection of reactivated chlamydia in our studies might also have required more than one or two passages for the detectable inclusions to form in culture. The simplest explanation of the failure of cortisone treatment to reactivate chlamydia in the present studies is, however, that chlamydia had really been eradicated from lungs, and that persistent infection no longer existed. This cannot be excluded, especially since we were not able to detect chlamydial mRNA in a similar model in study III, but PCR and histologic findings do not support this conclusion.

Previous animal models have shown the presence of chlamydial DNA for extended periods in mouse tissue samples, especially after reinoculations, and the lung inflammatory responses were also shown to persist for longer periods after repeated inoculations (Moazed et al. 1997). Therefore, our studies were not able to provide new data as evidence of a viable, persistent chlamydial infection in lung tissue in this mouse model.

6.3 Aortic changes caused by infections

A significant increase in aortic sinus lipid accumulation after six repeated C. pneumoniae inoculations in normocholesterolemic mice fed a chow diet with 0.2% cholesterol supplementation was shown in study III. Similarly, as seen in the levofloxacin experiments and in the telithromycin study (II), large lesion areas were detected in all study groups, including the SPG mock-inoculated mice. The lesion morphology was also similar in the uninfected and infected mice. The significant difference between the uninfected and repeatedly infected groups in study III was evidently due to the lack of smaller lesions in the infected group. This indicates that the atherosclerotic progress was perhaps faster in the mice inoculated several times with chlamydia. Similar absence of small lesions has not been reported in other studies, however, Ezzahiri and co-workers reported C. pneumoniae infection to accelerate the formation of more complex atherosclerotic lesions in ApoE3-Leiden mice (Ezzahiri et al. 2002). The exact mechanism by which C. pneumoniae induced the increased lipid accumulation was not assessed in these studies. The duration of cholesterol feeding had no significant effect on the lesion areas in our study, nor had the age at which the infections were started. These results, however, were obtained from the study groups inoculated only three times, and the results should be verified with four or more inoculations and with a longer follow-up time after the last inoculation. It has been suggested that C. pneumoniae invades pre-existing lipid lesions more easily than healthy aortic tissue (Hu et al. 1999), and infections of older mice with aortic lesions present might thus be more pathogenic. Other studies have reported that inoculations given at an older age increase the persistence of chlamydia (Moazed et al. 1997), the bacterial load and lung inflammation (Little et al. 2005) in mice. We were able to show elevated IgG levels against C. pneumoniae in the mice inoculated at an older age, whereas no differences in lung histopathology were seen in young vs. old inoculated mice (study III). Little and co-workers used BALB/c mice in their study, and this may explain the different histological findings, since these mice have been shown to respond to infection differently from C57BL/6 mice (Penttilä et al. 1998b).
As reviewed earlier, an atherogenic diet is needed in the development of advanced atherosclerosis in C57BL mice. The present results suggest that overt hypercholesterolemia is not necessary for *C. pneumoniae* infections to affect the vascular system and to increase subendothelial lipid accumulation in these mice. Yet, based on these results, we cannot say whether the increased lipid accumulation in the mice inoculated six times was really due to the persistent infection induced in the aortic sinus in study III, since we did not analyse the presence of chlamydia in aortic tissue. The theory of pathogen burden (Zhu *et al.* 2000) suggests that active pathogen-induced inflammatory responses in general have an important role in the development of atherosclerosis. The fact that several repeated inoculations led to areas with higher lipid accumulation in every mice in study III also supports this theory, although only *C. pneumoniae* was used as a pathogen to induce infection and inflammation. However, repeated *C. pneumoniae* inoculations are able to cause inflammatory changes in the aortic sinuses of normocholesterolemic mice (Blessing *et al.* 2000a) and endothelial dysfunction in ApoE-deficient hypercholesterolemic mice (Liuba *et al.* 2000). In study V, we detected altered aortic endothelial function after acute infection in C57BL/6J mice fed a normal chow diet, and the aortic tissue of these mice was also found to be positive for the presence of chlamydia by PCR. These studies support the view that not only systemic inflammatory responses but also local infection in aortic tissue is responsible for the increased aortic sinus lipid accumulation detected in our studies.

The altered endothelial function in study V was detected as a shift in the balance of endothelium-derived relaxing factors from NO towards vasorelaxing prostanoids. In uninfected aortas, the methacholine-induced relaxations were predominantly NO-dependent (blocked greatly by the NOS inhibitor L-NAME), while the blockage of prostanoid production by the COX inhibitor diclofenac did not modify the relaxations. However, in vessels from infected mice, the relaxations were inhibited by L-NAME but not statistically significantly, while diclofenac strongly and significantly impaired relaxations in the infected groups. Aortic relaxation in mice with *C. pneumoniae* infection was therefore markedly dependent on prostanoid-mediated vasodilatation. In addition to NO and vasorelaxing prostanoids, the presence of another endothelial relaxing factor, possibly endothelium-derived hyperpolarizing factor, was demonstrated in the normal uninfected aortas in the present study. This was shown by the remaining statistically significant relaxation in the presence of the combined blocking of both NO and prostanoids in the uninfected group compared to the chlamydia-infected groups, where basically no relaxation was seen. The effect of diclofenac, which indicated the role of vasorelaxing prostanoids in the aortic relaxation in C57BL/6J mice in our study differ from the results reported earlier in ApoE-deficient mice (Liuba *et al.* 2000). Liuba and co-workers suggested the presence of vasoconstricting prostanoids in the infected groups after both primary and repeated inoculations in the aortas of ApoE-deficient mice, since the blocking of cyclooxygenases in infected mice induced increased relaxation in their study. This discrepancy detected in these two animal models is not surprising, since the aortic responses and the susceptibility to atherosclerosis in ApoE-deficient mice is known to differ from that of C57BL/6J mice. The mutual superiority of the models cannot be judged on the basis of these results. However, C57BL/6J mice are free from the confounding effects of genetic manipulation on inflammatory responses, as discussed earlier.
Infection seemed to increase the expression of the inducible form of COX, COX-2, in the lung tissue of the mice in study V, but the study groups showed no differences in the 6-keto prostaglandin F1α metabolite levels measured from the serum samples. Previous \textit{in vitro} studies have shown a rapid induction of COX-2 expression after \textit{C. pneumoniae} infection within 24 hours p.i., which is then sustained at a lower level in human peripheral blood mononuclear cells (Rupp \textit{et al.} 2004). In study V, the samples were collected four days after infection, and it is possible that the peak of the response occurs earlier after the primary infection also \textit{in vivo}. We were not able to measure COX expression in aortic tissue in this study, to confirm the elevated levels indicated by the endothelial relaxation responses. Further studies are therefore needed to confirm our results.

Since infection might increase the prostanoid dependency of arterial relaxation mediated by endothelial cells, blocking of the function of cyclooxygenases could be expected to further impair arterial function. On the other hand, \textit{C. pneumoniae} infection was shown to increase COX-2-mediated induction of the proatherogenic prostaglandin E$_2$ and matrix metalloproteinase-1 production in peripheral blood mononuclear cells, which suggests that COX inhibition might actually be beneficial in view of atherosclerotic events (Rupp \textit{et al.} 2004). Therefore, the COX-mediated responses seem to be two-sided, and the effects of both selective and unselective COX inhibitors on atherosclerotic development induced by infection might be worth studying. Previously, it has been shown that selective COX-2 inhibition does not affect atherosclerotic development in ApoE-deficient mice (Bea \textit{et al.} 2003); it would be interesting to study the effect of chlamydial infection in this model as well as in wild-type mice.

### 6.4 Effects of treatments

The \textit{in vitro} susceptibility of the isolate K7 to telithromycin in our study was in agreement with the values obtained in an earlier study with several \textit{C. pneumoniae} isolates (Roblin & Hammerschlag 1998a). The MIC of levofloxacin detected in the present study was also within the range reported earlier, but the MBC of levofloxacin was found to be slightly higher (Miyashita \textit{et al.} 1997). This difference is most probably due to the different cell culture model used. Telithromycin treatment was effective in eliminating culture positivity from lung tissue homogenates and decreased the inflammatory reactions in lungs after acute intranasal \textit{C. pneumoniae} inoculation in study I. However, lung tissue remained PCR-positive in 10% of these culture-negative mice similarly to the previous mouse models of \textit{C. pneumoniae} inoculation treated with different antibiotics (Malinverni \textit{et al.} 1995b, Masson \textit{et al.} 1995, Bin \textit{et al.} 2000).

Similar results were also obtained after repeated inoculations in study II. Although treatment with telithromycin after both of the two inoculations was most effective in the eradication of chlamydia, the PCR results showed persistent presence of chlamydial DNA in some of the mice. Moderate lung inflammation also persisted in these mice despite the treatment. Immediate treatment after both inoculations also significantly decreased aortic sinus lipid accumulation compared to the group treated only after the second inoculation. Telithromycin given with delay, after three repeated inoculations, had no effect at all on
the persistence of chlamydial DNA in lung tissue, and no significant difference in aortic lipid accumulation between the treated group and the placebo group was found, either. Similarly, in the levofloxacin experiments, oral treatment with either levofloxacin or erythromycin for seven days after the third inoculation was not able to decrease the lipid accumulation areas compared to the placebo-treated mice. Thus, in our studies, delayed treatments were not effective in preventing the development of atherosclerotic changes, which is also in agreement with the previous animal models with *C. pneumoniae* infections. In ApoE-deficient mice inoculated twice and treated with azithromycin for either two and three weeks (Rothstein *et al.* 2001) or six weeks (Blessing *et al.* 2005) after the last inoculation, *C. pneumoniae* infection increased the aortic Oil-Red O-stained lesion areas in the infected mice, but azithromycin did not reduce their size. Similar results have also been obtained from rabbit models, in which delayed treatment with azithromycin had no effect (Fong *et al.* 1999a), and delayed treatment with clarithromycin only slightly diminished the atherosclerotic development (Fong *et al.* 2002).

In the levofloxacin experiments, erythromycin and levofloxacin were effective in decreasing the persistence of IgG autoantibodies against mHsp60 protein: 12 weeks after the third inoculation, at about ten weeks from the end of the treatment, significant reduction in mHsp60 IgG levels was still detected. Both macrolide and fluoroquinolone antibiotics have been shown to possess anti-inflammatory activities (Scaglione & Rossoni 1998, Azuma *et al.* 1999), and our studies indicate that, despite the insufficient effects of these antibiotics on chlamydia eradication, they might significantly attenuate the formation of autoimmune responses against self-Hsp60.

The findings of the present and previous *C. pneumoniae* treatment studies are in accordance with the results from large antibiotic treatment trials in humans. Prolonged treatment of patients with cardiovascular disease for several months (O'Connor *et al.* 2003) or even for one year (Grayston *et al.* 2005) with antibiotics effective against chlamydia were not able to decrease the cardiovascular end point events evaluated in these studies. These disappointing results are, however, understandable in view of the reported research data. Assuming that chlamydia or other microbial infections promote atherosclerotic development via eliciting systemic inflammatory responses, i.e. causing a pathogen burden, antimicrobial treatment at the advanced disease state is obviously useless. Still, most of the intervention trials were targeted to treat chronic *C. pneumoniae* infection in the atherosclerotic vessels, based on the wide variety of data supporting the presence of chlamydia in these tissues. In this light, the results support the earlier in vitro findings, which suggest that persistent chlamydia infection does not respond to antimicrobial treatment (Oriel & Ridgway 1982, Kutlin *et al.* 1999, Gieffers *et al.* 2001, Baltch *et al.* 2004, Reveneau *et al.* 2005). Still, analysis of subpopulations in the WIZARD study (O'Connor *et al.* 2003) indicated that diabetic, smoking male patients gained significantly from the treatment. Furthermore, the trials in which the treatment was started within 48 hours after an acute cardiac event showed positive impacts of the treatment on the cardiovascular end points (Gurfinkel *et al.* 1997, Stone *et al.* 2002, Sinisalo *et al.* 2002). These observations suggest a need for methods able to identify the patients who might benefit from antibiotic treatments. One additional factor possibly influencing the results of the recent large-scale treatment trials is the prevalent
concomitant use of statins, which have been shown to have both anti-inflammatory and antimicrobial potential.

Although, expectedly, not equally effective as the antibiotic drugs tested, luteolin treatment given prior to and during acute *C. pneumoniae* infection was, nevertheless, able to decrease both the presence of infectious chlamydia and the inflammatory response in lung tissue in study IV. Previous reports have shown numerous plant-derived phenolic compounds, including luteolin, quercetin and octyl gallate, to be microbicidal in vitro (Kaul *et al.* 1985, Rauha *et al.* 2000, Xu & Lee 2001) and also to possess strong anti-inflammatory potential, as the compounds tested in study IV (Murase *et al.* 1999, Xagorari *et al.* 2001, Kotanidou *et al.* 2002, Cho *et al.* 2003). Antimicrobial and anti-inflammatory effects of these compounds have been associated with their ability to bind bacterial DNA and gyrase (Plaper *et al.* 2003) and to inhibit proinflammatory cytokine production by suppressing the phosphorylation of nuclear factors, such as NF-κB (Xagorari *et al.* 2001, Cho *et al.* 2003). In addition, quercetin and luteolin have been shown to induce apoptosis in human leukaemia HL-60 cells with luteolin being a more potent inducer than quercetin (Cheng *et al.* 2005). All these mechanisms may have contributed to the suppressing effects of luteolin detected in study IV, but the results from treatment with quercetin are inconsistent with most previous reports. Quercetin and octyl gallate were given to mice in tenfold doses compared to luteolin, which was based on the previous reports and on the in vitro MIC results (Alvesalo *et al.* in press). A few earlier studies have shown that, at some point, an increased concentration of either quercetin or luteolin actually has inverse effects on the outcome of infection in vivo (Veckenstedt *et al.* 1985, Kotanidou *et al.* 2002). The pharmacokinetics and bioavailability of different phenolic compounds may vary considerably (Manach *et al.* 2004), and it is possible that the given dose of quercetin was too high in study IV. The bioavailability of luteolin at the inflammatory foci might also be better than that of quercetin, since it has been shown that free luteolin aglycone can be found in plasma together with its main metabolite, luteolin monoglucuronide. Luteolin monoglucuronide was also shown to be hydrolysed to free luteolin by the β-glucuronidase of neutrophils at the site of inflammation (Shimoi *et al.* 2001). The conjugated forms of flavonoids, consisting mainly of glucuronides, are not as efficient as the unconjugated compounds in view of the beneficial effects of flavonoids detected in vitro (Spencer *et al.* 2003, Manach *et al.* 2004). No detectable levels of aglycone forms of the studied compounds were found from the serum samples of the mice in the present study, when the samples were collected one hour after intraperitoneal administration. Finally, the effects of octyl gallate treatment were neither positive nor negative in our study.
7 Conclusions

Normocholesterolemic wild-type mice were used to investigate the effects of acute and repeated *C. pneumoniae* infections on the development of chronic infection and atherosclerotic changes in these studies. The effectiveness of the antimicrobial agents telithromycin, levofloxacin and erythromycin and the phenolic compounds quercetin, luteolin and octyl gallate were also tested in treating the infection and preventing its sequelae. The mouse model employed proved generally suitable for these purposes. However, we were not able to reactivate persistent lung infection or to detect viable chlamydia after multiple inoculations in mouse lung tissue, although inflammation and chlamydial DNA were shown to persist there.

Repeated *C. pneumoniae* infections were shown to significantly increase aortic sinus lipid accumulation in the mice fed a normal chow diet supplemented with 0.2% cholesterol. In addition, acute *C. pneumoniae* infection was able to affect endothelial function and to shift the balance of the vasorelaxing factors of aortic endothelium towards prostanoid dependency in these mice. A significant correlation between the IgG antibodies against chlamydial and mouse Hsp60 was observed, and both of these antibody levels also correlated with the severity of the lung inflammatory response induced by infections. These findings support the role of *C. pneumoniae* in the development of atherosclerosis and the induction of an autoimmune response against Hsps after chlamydial infection.

Treatment of *C. pneumoniae* infection with a ketolide, fluoroquinolone, and macrolide antibiotics abolished the viable chlamydia from mouse lungs effectively. The novel ketolide antibiotic, telithromycin, also significantly decreased aortic sinus subendothelial lipid accumulation if given after each inoculation. Delayed treatment after repeated inoculations with any of the antimicrobial agents had no effect on lipid accumulation. Complete eradication of PCR positivity was not achieved with these treatment regimens, either.

The inability of common antibiotic agents to treat and eradicate persistent chlamydial infections warrants search for new methods and compounds effective against chlamydia, especially since the development of chlamydial vaccines has proven difficult. The beneficial antichlamydial and anti-inflammatory effects of a flavonoid luteolin on acute *C. pneumoniae* lung infection in vivo were promising and encouraged the search for even
more potent compounds. Whether any treatment can prevent the spreading of chlamydia from lungs to other tissues should also be assessed in the further studies.
References


*Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic 

subinhibitory concentrations induce persistence of *Chlamydia pneumoniae*. Antimicrob Agents 
Chemother 48: 1402-5.

*Chlamydia pecorum* and *Chlamydia pneumoniae* cause infections in koalas. Syst Appl 


wall cells to infection with *Chlamydia pneumoniae*. J Clin Microbiol 33: 2411-4.

Gordon EM & Blumer JL (2004) Rationale for single and high dose treatment regimens with 

Grayston JT, Kuo CC, Wang SP & Altman J (1986) A new *Chlamydia psittaci* strain, TWAR, 

Grayston JT, Kuo CC, Campbell LA & Wang SP (1989a) *Chlamydia pneumoniae* sp. nov. for 

Grayston JT, Mordhorst C, Bruu AL, Vene S & Wang SP (1989b) Countrywide epidemics of 

Grayston JT, Campbell LA, Kuo CC, Mordhorst CH, Saikku P, Thom DH & Wang SP (1990) A 

675-84.

Grayston JT, Krommal RA, Jackson LA, Parisi AF, Muhlestein JB, Cohen JD, Rogers WJ, Crouse 
JR, Borrowdale SL, Schron E & Knirsch C (2005) Azithromycin for the secondary prevention 


*Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in male survivors 

roxithromycin in non-Q-wave coronary syndromes: ROXIS Pilot Study. ROXIS Study Group. 

Gurfinkel E, Bozovich G, Beck E, Testa E, Livellara B & Mautner B (1999) Treatment with the 
antibiotic roxithromycin in patients with acute non- Q-wave coronary syndromes. The final 


Hahn DL, Dodge RW & Golubiatnikov R (1991) Association of *Chlamydia pneumoniae* (strain 
TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. JAMA 266: 
225-30.

Hahn DL (1999) *Chlamydia pneumoniae*, asthma, and COPD: what is the evidence? Ann Allergy 

pathogen. Front Biosci 7: 66-76.


Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. Circulation 97: 633-6.


