Taina Lajunen

PERSISTENT CHLAMYDIA PNEUMONIAE INFECTION, INFLAMMATION AND INNATE IMMUNITY
TAINA LAJUNEN

PERSISTENT CHLAMYDIA PNEUMONIAE INFECTION, INFLAMMATION AND INNATE IMMUNITY

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Oulu, Finland

**Abstract**

*Chlamydia pneumoniae* is an obligatory intracellular pathogen that causes upper and lower respiratory tract infections. Like other *Chlamydial* species, also *C. pneumoniae* has a tendency to cause persistent infections, which have been associated with different cardiovascular, neurological, and respiratory diseases. In addition, a few studies have reported an association between *C. pneumoniae* seropositivity and an elevated body mass index (BMI), and it has been shown that *C. pneumoniae* is capable of infecting preadipocytes and adipocytes. The main aims of this study were to study if certain gene polymorphisms regulate the serum levels of innate immunity and inflammation proteins, and if the polymorphisms are associated with markers of *C. pneumoniae* infection; to compare different methods in detection of *C pneumoniae* in atherosclerotic tissue; and to study if serum levels of chlamydial LPS (cLPS) are associated with BMI.

The serum levels of inflammatory and innate immunity markers, namely interleukin 6 (IL-6), C-reactive protein (CRP), LPS-binding protein (LBP), and soluble CD14, in apparently healthy individuals were found to correlate with each other and possibly be regulated by the polymorphisms of genes important in inflammation and innate immunity. Especially the serum LBP levels may be regulated by the *LBP* (rs2232618) and *toll-like receptor 4* (rs4986790) polymorphisms. The *IL-6* (rs1800795) polymorphism was found to be associated with *C. pneumoniae* antibody positivity.

*C. pneumoniae* DNA and cLPS could be found from atherosclerotic tissue. A new, cLPS enzyme immunoassay method was developed in this study, and it might provide a standardized, commercial method for the detection of chlamydia in tissue samples, if the sensitivity of the method could be increased e.g. by testing multiple pieces of tissue. *In situ* hybridization method was found to be complicated by technical problems and the repeatability of polymerase chain reaction was poor.

*C. pneumoniae* IgG positivity and elevated serum cLPS and CRP levels were associated with an elevated BMI. There was also a strong association between cLPS levels and inflammation as measured by CRP levels. The lack of association between serum total endotoxin activity and BMI implies that the association between infection and an elevated BMI may be specific to certain pathogens.

**Keywords:** body mass index, cardiovascular diseases, *Chlamydia pneumoniae*, genetic polymorphism, inflammation, lipopolysaccharides, natural immunity
To my family
Acknowledgements

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAA</td>
<td>abdominal aortic aneurysm</td>
</tr>
<tr>
<td>AOD</td>
<td>occlusive aortic disease</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CAR</td>
<td>symptomatic carotid stenosis</td>
</tr>
<tr>
<td>CD14</td>
<td>cluster of differentiation 14</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>cLPS</td>
<td>chlamydial LPS</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EB</td>
<td>elementary body</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>GE</td>
<td>genome equivalent</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>hsCRP</td>
<td>high-sensitivity C-reactive protein</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat-shock protein</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>inter-cellular adhesion molecule</td>
</tr>
<tr>
<td>IEM</td>
<td>immuno-electron microscopy</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IQR</td>
<td>inter-quartile range</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridization</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-binding protein</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MBL</td>
<td>mannose-binding lectin</td>
</tr>
<tr>
<td>mCD14</td>
<td>membrane-bound CD14</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MD</td>
<td>median value</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIF</td>
<td>microimmunofluorescence</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MOMP</td>
<td>major outer membrane protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NA</td>
<td>not applicable</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>nuclear factor-κβ</td>
</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Nod</td>
<td>nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NS</td>
<td>not statistically significant</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</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 RNA</td>
</tr>
<tr>
<td>sCD14</td>
<td>soluble CD14</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>totLPS</td>
<td>total endotoxin activity</td>
</tr>
<tr>
<td>T3S</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell-adhesion molecule 1</td>
</tr>
</tbody>
</table>
List of original publications


*Lajunen T née Korhonen T

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1 Introduction

*Chlamydia pneumoniae* is an obligatory intracellular pathogen that causes upper and lower respiratory tract infections. Most adults have been exposed to this pathogen at some time during their life, and the its seroprevalence increases with age due to e.g. recurring infections or reactivation of chronic infections. (Saikku 1992.) Like other *Chlamydial* species, also *C. pneumoniae* has a tendency to cause persistent infections (Hogan *et al.* 2004), which have been associated with diseases like coronary heart disease, stroke, abdominal aortic aneurysm, Alzheimer’s disease, asthma, and reactive arthritis (Saikku 2002). *In vitro*, persistent infection can be triggered by interferon γ, antibiotics, and depletion of nutrients (Hogan *et al.* 2004).

Elevated levels of serological *C. pneumoniae* infection markers are associated with cardiovascular diseases (CVDs) (Saikku *et al.* 1988), and *C. pneumoniae* is capable of infecting and surviving in several cell types important in pathogenesis of CVD (Gaydos 2000). The presence of the pathogen in atherosclerotic tissue has also been shown in both animal models and humans using several different methods. However, no validated method for detecting persistent infections exists, and the positivity rates of PCR studies of atherosclerotic tissue samples, in particular, vary greatly. (Watson & Alp 2008.) This may be due to the small amount and patchy distribution of *C. pneumoniae* DNA in atherosclerotic tissue (Cochrane *et al.* 2003), but also to differences in practices and methods and methodological problems (Apfalter *et al.* 2002).

In obesity, increased infiltration and activation of macrophages in adipose tissue is seen, and adipose tissue also secretes inflammatory cytokines. In addition, elevated serum levels of inflammatory markers are associated with a higher body mass index (BMI) and obesity. Increased levels of inflammatory markers again may contribute to development of obesity and other inflammation-related conditions like decreased insulin sensitivity, type 2 diabetes, metabolic syndrome, and CVD, which are all associated with obesity, as well. (Cancello & Clément 2006.) A few studies have reported an association between seropositivity for *C. pneumoniae* and a elevated BMI (Karppinen *et al.* 2003, Saikku *et al.* 2004). Two recent studies have also shown that *C. pneumoniae* is capable of infecting preadipocytes and adipocytes (Bouwman *et al.* 2008, Shi *et al.* 2008).

Several studies have addressed an association of variation in genes important in innate immunity and inflammation with susceptibility to e.g. infections and CVD. Such genes which may also play a role in susceptibility to developing
persistent *C. pneumoniae* infection and/or in modifying the effect of *C. pneumoniae* on the pathogenesis of several chronic diseases, are e.g. lipopolysaccharide (LPS)-binding protein (LBP), CD14, toll-like receptor 2 (TLR-2), TLR-4, and interleukin 6 (IL-6) (Arbour *et al.* 2000, Lorenz *et al.* 2000, Hubacek *et al.* 2001, Veres *et al.* 2002).
2 Review of the literature

2.1 Chlamydia pneumoniae

2.1.1 History

Due to their small size and obligate intracellular nature, chlamydiae were originally thought to be viruses. Based on the work by Moulder in the mid-1960s, it was concluded that agents causing trachoma, psittacosis, and related diseases were indeed bacteria and distinct from viruses. It was also concluded on the basis of their morphology, developmental cycle, and group antigen that these organisms belong to one genus, called Chlamydia after the suggestion of Jones, Rake, and Stearns in 1945. Based on the appearance of the inclusion, the ability of the strains to produce detectable glycogen, and their sensitivity to sodium sulfadiazine, these organisms were further classified into two species, C. trachomatis and C. psittaci. (Page 1968.)

*C. pneumoniae* was first isolated in 1965 in Taiwan from the eye of a child (TW-183), and at that time the isolate was thought to be a strain of *C. trachomatis*. Along with the availability of cell culture techniques for chlamydiae in 1971, TW-183 was classified as a serologically different, new group of *C. psittaci*. (Saikku et al. 1985.) In 1980, Darougar et al. reported that, in apparently healthy London blood donors, 14% of the women and 24% of the men had IgG antibodies against IOL-207, an atypical chlamydial isolate from the eye of an Iranian child (Darougar et al. 1980), said to be similar to TW-183 (Dwyer et al. 1972). In 1985, an association between an etiologic agent similar to TW-183 and mild pneumonia cases that occurred in northern Finland in 1978 with no known avian source, was shown by microimmunofluorescence (MIF) test (Saikku et al. 1985). The next isolates were obtained in Washington in 1983 to 1986 from pharyngeal swabs of adults with acute respiratory diseases (Grayston et al. 1986, Kuo et al. 1986). The isolate AR-39 was the first respiratory isolate and it was obtained from a university student with pharyngitis in 1983 (Grayston et al. 1986). This organism was given the name *C. psittaci* strain TWAR, according to the laboratory names of the first two isolates TW-183 and AR-39 (Kuo et al. 1986). The strain TWAR was classified as a new species of the genus Chlamydia in 1989, and it was named *C. pneumoniae* (Grayston et al. 1989).
2.1.2 Taxonomy

In 1999, a new taxonomy was proposed for the order Chlamydiales based on 16S and 23S rRNA comparisons; it included two new families, Parachlamydiaceae and Simkaniaceae to contain the recently identified chlamydia-like species (Kahane et al. 1995, Amann et al. 1997), and divided the family Chlamydiaceae into two genera Chlamydia and Chlamydophila. The genus Chlamydia contained C. trachomatis, C. muridarum, and C. suis whereas C. pneumoniae, C. pecorum, C. psittaci, and the three new species (C. abortus, C. caviae, and C. felis) derived from C. psittaci, formed the new genus Chlamydophila (Everett et al. 1999) (Table 1).

Table 1. New Chlamydiales taxonomy as proposed by Everett et al.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydiaceae</td>
<td>Chlamydia muridarum</td>
<td>mouse, hamster</td>
</tr>
<tr>
<td></td>
<td>Chlamydia suis</td>
<td>swine</td>
</tr>
<tr>
<td></td>
<td>Chlamydia trachomatis</td>
<td>human</td>
</tr>
<tr>
<td></td>
<td>Chlamydophila abortus</td>
<td>mammals</td>
</tr>
<tr>
<td></td>
<td>Chlamydophila caviae</td>
<td>guinea pig</td>
</tr>
<tr>
<td></td>
<td>Chlamydophila felis</td>
<td>cat</td>
</tr>
<tr>
<td></td>
<td>Chlamydophila pecorum</td>
<td>mammals</td>
</tr>
<tr>
<td></td>
<td>Chlamydophila pneumoniae</td>
<td>human, horse, koala</td>
</tr>
<tr>
<td></td>
<td>Chlamydophila psittaci</td>
<td>birds</td>
</tr>
<tr>
<td>Simkaniaceae</td>
<td>Simkania negevensis</td>
<td>human</td>
</tr>
<tr>
<td>Parachlamydiaceae</td>
<td>Parachlamydia acanthamoebae</td>
<td>amoebae</td>
</tr>
</tbody>
</table>

Modified after Everett et al. (1999).

This new taxonomy has not generally been accepted among chlamydiologists; especially the division into two genera, based on minor differences in the 16S and 23S rRNA sequences, was criticized (Schachter et al. 2001). Also, using a less than > 95% identity as a division criteria was considered inappropriate for intracellular organisms that are not fast-growing and do not commonly exchange DNA. Now, when the genomic sequences of several chlamydial species and strains are available, it has become apparent that there is an almost 80% conservation of genes and gene order between C. abortus, C. trachomatis, and C. pneumoniae, whereas in free-living bacteria it is common that almost no discernable conservation is seen in gene order beyond the operon between strains of the same species (Stephens 2008). The new taxonomy has also brought confusion to the field and two different names for C. pneumoniae can be found in
publications. However *Chlamydia pneumoniae* is more widely used than *Chlamydophila pneumoniae*, and it has been proposed that “a one-genus, multiple-species” system should again be adopted (Stephens 2008) (Fig. 1). Based on these arguments, the name *Chlamydia pneumoniae* is used in this thesis and in the original publications.

**Fig. 1.** *Chlamydiaceae* taxonomy proposed by Stephens. The length of the lines does not represent evolutive distance. Modified after Stephens (2008).

### 2.1.3 Developmental cycle

*C. pneumoniae* is an obligatory intracellular pathogen that has a two-phase developmental cycle typical to chlamydial species (Fig. 2). Small (a diameter of approximately 0.3 μm) and metabolically inert elementary bodies (EB) are the infectious form. The typical chlamydial EB is round and has a very little periplasmic space. *C. pneumoniae* EBs may, however, sometimes be pear-like and contain a large periplasmic space (Grayston *et al.* 1989, Popov *et al.* 1991, Miyashita *et al.* 1993). EBs attach to the cell surface and then enter the cell through receptor-mediated endocytosis in clathrin-coated pits, pinocytosis in non-clathrin-coated pits, or by phagocytosis (Wyrick 2000). The receptors used for entry may be different for different chlamydial species. It has been suggested that, to enter and infect endothelial cells, *C. trachomatis* may use a mannan receptor and *C. pneumoniae* mannose a 6-phosphate/insulin-like growth factor 2
(M6P/IGF2) receptor (Puolakkainen et al. 2005). Inside the host cell, EBs inhibit fusion of phagosome with lysosome and instead associate with exocytic vesicles. For C. trachomatis, homotypic endosomal fusion results in one vacuole with several EBs, whereas C. pneumoniae and C. psittaci infections result in several inclusions per host cell (Wyrick 2000). In the early phases of infection, chlamydiae secure their intracellular growth by inhibiting apoptosis (Peters et al. 2007). EBs then transform into larger (diameter ~1.0 μm) reticulate bodies (RB), which are the replicative form. The RBs then begin to divide by binary fission and an inclusion 2–12 μm in diameter, consisting of up to thousands of bacteria, is formed. The shape of C. pneumoniae inclusions in HeLa cells is oval and dense (Kuo et al. 1986). Growing chlamydiae acquire amino acids, nucleotides, and lipids from the pools of the host cell. Membrane lipids like glycerophospholipids, sphingolipids, and cholesterol are acquired by selectively rerouting Golgi-derived exocytic vesicles and multivesicular bodies. It was recently shown that C. trachomatis also transports cytoplasmic lipid droplets into inclusions, possibly by a mechanism dependent on a chlamydial secreted protein Lda3 and an inclusion membrane protein IncA. (Cocchiaro et al. 2008.) In the late phase of the developmental cycle, different forms of chlamydia can be found in the inclusion: dividing RBs, intermediate forms, and EBs (Hatch 1999). At the end of the reproductive cycle, EBs are again formed and they escape from the cell by host cell lysis or exocytosis. Chlamydiae may also enter into a persistent phase where the RBs or aberrant bodies (AB) are viable but abnormal looking, non-dividing, and their metabolic activity is low. This persistence may be triggered by cytokines like IF-γ, by antibiotic treatment, or by depletion of certain nutrients (Hogan et al. 2004).
2.1.4 Structure and molecules important in pathogenesis

Peptidoglycan

Chlamydiae are Gram-negative bacteria, and in all stages of development, chlamydial cells appear to have inner and outer membranes. However, unlike other Gram-negative bacteria, chlamydia may not have a peptidoglycan layer in its periplasmic space, since muramic acid, a component of eubacterial peptidoglycan, has not been biochemically detected in chlamydiae or it has only been detected in small amounts (Fox et al. 1990). It has been suggested that instead of peptidoglycan, a disulfide bond cross-linked outer membrane and periplasmic proteins may be responsible for the osmotic integrity of EBs, and that the absence of these bondings in RBs makes them osmotically fragile (Hatch 1999). However, RBs are sensitive to penicillin (Matsumoto & Manire 1970), a feature that usually marks the presence of peptidoglycan, and at least most of the genes needed for peptidoglycan assembly are present in the genome of C. trachomatis serovar D (Stephens et al. 1998) and also in C. pneumoniae.
CWLO29 (Kalman et al. 1999). It was recently shown that cytosolic nucleotide-binding oligomerization domain 1 (Nod1), which detects diaminopimelate (DAP)-containing peptidoglycan, mostly found in Gram-negative bacteria, may be important in C. pneumoniae-induced cell activation (Welter-Stahl et al. 2006), and that chlamydia can synthesize DAP-containing peptidoglycan (McCoy et al. 2006). Thus, it has been proposed that some amount of peptidoglycan is present in chlamydial cells. (Pavelka 2007.)

Lipopolysaccharide

Lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria, is, in general, one of the most potent stimulators of innate immunity. The part of LPS responsible for endotoxin activity is a hydrophobic phosphoglycolipid called lipid A. Lipid A is connected to the core unit of LPS, which can be further divided into an inner core and an outer core. The structurally most conserved part of LPS is lipid A together with the 3-deoxy-D-manno-octulosonic (Kdo)-containing inner core. Attached to the outer core may be O polysaccharide (O antigen). Chlamydial LPS (cLPS) can be phenotypically classified as rough LPS, since it does not contain an O antigen. (Rietschel et al. 1994, Raetz & Whitfield 2002.) One study has reported the presence of smooth-type LPS in chlamydia grown in a yolk sac (Lukáčová et al. 1994), but this finding has not been confirmed. The genus-specific (or family-specific if Chlamydia and Chlamydophila are used) epitope of cLPS is located in the trisaccharide of Kdo, and it contains a unique 2–8 linkage of two Kdo residues (Brade et al. 1987). Studies on cLPS have shown that its biological activity is lower than that of enterobacterial LPS. C. psittaci LPS was shown not to be pyrogenic or to cause Schwartzman reactivity in rabbits, and it was not toxic to galactosamine-sensitized mice (Brade et al. 1987). C. trachomatis LPS was shown to induce tumor necrosis factor-α (TNF-α) production in whole blood with approximately 100-fold less potency than LPS of Salmonella minnesota or Neisseria gonorrhoeae (Ingalls et al. 1995). In the study by Heine et al. (2003), a minimum of 10 ng/ml of C. trachomatis LPS was needed for activation of TNF release from human mononuclear cells, and an amount of 100 ng/ml resulted in a similar level of activation as 1.0 ng/ml of enterobacterial LPS. In the same study, C. psittaci LPS was shown to be approximately even tenfold less active than LPS of C. trachomatis. This reduced activity may be due to the unusual structure of chlamydial lipid A, which contains only five fatty acids and has long fatty acyl
chains that contain up to 22 carbon atoms (Qureshi et al. 1997). The low toxicity of cLPS may be an adaptation for intracellular persistence of chlamydiae. Despite its low toxicity, cLPS is capable of inducing translocation of nuclear factor-κβ (NF-κβ) in CHO/CD14 reporter cells, although at lower levels than enterobacterial LPS (Heine et al. 2003). It has recently been suggested that the lower toxicity of cLPS may be explained by its lower affinity to LPS recognition molecules like LBP and CD14 (Tsutsumi-Ishii et al. 2008).

Major outer membrane protein

The chlamydial protein present in the largest quantity in EBs and RBs is a major outer membrane protein (MOMP), and it is coded (OmpA) by all chlamydiae. In EBs, MOMP is extensively disulfide cross-linked, whereas in RBs it is non-cross-linked. Most likely, MOMP is involved in adhesion of chlamydia to the host cell. It may serve as an initial site of interaction by providing electrostatic and hydrophobic binding sites or by a more specific interaction with host cell receptors (Su et al. 1990). Four variable sequences, surrounded by more conserved areas, are found in MOMP, and these variable areas tend to be surface-exposed. MOMP shows antigenic variation between the different C. trachomatis strains, but this variation may be a result of host immune pressure rather than a pathogenicity characteristic (Brunham et al. 1993). MOMP, probably arranged as trimers, also functions as a porin, through which e.g. adenosine triphosphate (ATP) can be passaged (Wyllie et al. 1998). Unlike in C. trachomatis and C. psittaci, C. pneumoniae MOMP has been reported not to be the immunodominant antigen (Campbell et al. 1990a), perhaps as a result of masking by other proteins or because immune response is targeted against conformational epitopes seen in trimeric MOMP (Hatch 1999).

Polymorphic membrane proteins

The family of polymorphic membrane proteins (Pmps), predicted to encode outer membrane proteins that may affect chlamydial virulence, is relatively large in the C. pneumoniae genome (21 members) when compared with the genome of C. trachomatis (nine members). This protein family alone represents 22% of the increased coding capacity of C. pneumoniae (in the C. pneumoniae genome there are 187711 additional nucleotides when compared with C. trachomatis) (Kalman et al. 1999). These genes show remarkable diversity (< 50% amino acid sequence
identity), but they share conserved amino acid repeats in their aminoterminal half (Grimwood et al. 2001). It seems that all of these pmp genes are transcribed; however, it may be that not all of them are translated. There may also be differences in the expression (pmpG, pmpG3, and pmpG10) and size (pmpG6) of Pmps between the studied strains, CWL029 and AR29, and it has been suggested that this variation is important for the function of these proteins (Grimwood et al. 2001). At least Pmp20 and Pmp21 of C. pneumoniae have been shown to stimulate the NF-κβ pathway and production of IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1) in human endothelial cells in vitro (Niessner et al. 2003). While the function of most Pmps remains to be solved, it has been suggested that Pmp10, the most highly expressed pmp in C. pneumoniae, is closely associated with MOMP, and its function may be to protect the C terminal part of MOMP from proteolytic cleavage (Juul et al. 2007).

**Type III secretion system**

Approximately 13–30 hollow projections extend from the cytoplasmic membrane of chlamydiae through the outer membrane, the inclusion membrane, and into the host cell cytoplasm. In EBs, these projections are arranged in a hexagonal array, but when the EBs transform into RBs these projections become randomly distributed. (Wyrick 2000.) These projections are known as Matsumoto’s projections, according to their finder, and are thought to be part of the chlamydial type III secretion (T3S) system (Bavoil & Hsia 1998). The type III secretion system, through which bacterial virulence factors are transported to the eukaryotic cytosol (vir-T3S), has been found from several pathogenic Gram-negative bacteria, e.g. *Yersinia, Salmonella, Shigella*, pathogenic *Escherichia coli*, and *Chlamydiales*, including environmental species like *Simkania negevensis*. *Chlamydiaceae* also possess several genes coding for the flagellar type III secretion system (fla-T3S). However, the meaning of these genes is not clear, since chlamydia only seems to have part of the genes needed for functional flagella. The injectisome of chlamydia is thought to be composed of several proteins (some of them putative): a lipoprotein called CDsJ, integral membrane proteins (CDsR-V), an inner membrane protein (CDsD), and an outer membrane ring protein (CDsC). A chlamydial outer protein called CopB, detectable in the inclusion membrane, has been proposed to function as an entry point for the T3S needle and to facilitate translocation of the secreted proteins into the eukaryotic cytosol. The most likely candidates for secreted effector proteins include the
translocated actin-recruiting phosphoprotein (Tarp), inclusion membrane proteins (IncA, IncB, and IncC), and CopN, a protein similar to *Yersinia* T3S regulator called YopN. (Peters *et al.* 2007.) Based on the fact that the size of the projection patches decreases during late differentiation, possibly due to decreasing contact of RB with the inclusion membrane as a result of growth, it has been suggested that they may play a role in initiating late differentiation of an RB to an EB. Possibly, the T3S system is turned off at some point and this then provokes late differentiation, which may be facilitated by detachment from the inclusion membrane. (Peters *et al.* 2007.)

**Heat shock proteins**

Heat shock proteins (Hsps) are expressed as a response to different physical, chemical, or biological stressors like heat, UV light, xenobiotics, drugs, and infections, but they also take part in normal cell functions. Many Hsps act as chaperones of proteins that have been e.g. misfolded or damaged, and thus protect cells from possible cytotoxic effects caused by them. Hsps are produced by a large variety of different organisms from prokaryotes to eukaryotes, and inside the Hsp families there is a high degree of conservation between the different organisms. (Lindquist & Craig 1988, Wu & Tanguay 2006.) In chlamydiae, genes encoding chlamydial Hsps (cHsps), cHsp10 (GroES), cHsp60 (GroEL), and cHsp70 (DnaK) are present (Engel *et al.* 1990, LaVerda & Byrne 1997). In *C. trachomatis* infections, cHsp60 and cHsp70 are among the major serologically defined antigens, with 50% and 45% antibody seroprevalence, respectively (Brunham & Peeling 1994). Chlamydial Hsp60 and Hsp70 are expressed constitutively; the proteins are present in both EBs and RBs, and during heat stress an increase in their expression is seen (Brunham & Peeling 1994). During interferon-γ (IFN-γ)-induced persistent *C. trachomatis* infection, cHsp60 is produced in considerably increased ratios to MOMP (5:1) when compared with normal infection (~1:1) (Beatty *et al.* 1993). In contrast, upregulation of both cHsp60 and MOMP as a response to IFN-γ treatment in *C. pneumoniae* has been reported (Molestina *et al.* 2002).

**Nucleoid**

When examined with an electron microscope, an electron-dense nucleoid can be seen in chlamydial EBs and intermediate forms, but not in RBs. The complete
composition of the nucleoid is unclear, but likely it contains histone-like proteins Hc1 and Hc2. The genes coding for these proteins (hctA and hctB, respectively) show a significant degree of similarity with eukaryotic H1-type histones, and they both bind DNA in vitro. Several differences in the DNA binding between Hc1 and Hc2 have been observed, and it has been suggested that Hc1 is mainly involved in DNA compacting in EBs and that Hc2 may play a smaller role in nucleoid morphology and possibly take part in regulation of stage-specific gene expression. (Perara et al. 1992, Kaul & Wenman 1998, Hatch 1999.)

2.1.5 Infections

**Seroepidemiology**

In a primary *C. pneumoniae* infection, IgM antibody response develops approximately 2–3 weeks after infection, and after 2–6 months it usually cannot be detected anymore. IgG and IgA responses develop more slowly and may reach high titres only 6–8 weeks after infection and may then remain elevated for long periods. Especially low titres may persist for several years. However, due to the shorter half-life of IgA antibodies, IgA positivity disappears faster than IgG positivity. IgG and IgA responses are faster in reinfections and can often be detected within 1–2 weeks after infection, whereas IgM response may not develop. (Grayston et al. 1990, Falck et al. 1994, Dowell et al. 2001, Paldanius et al. 2005.)

*C. pneumoniae* is found worldwide, and seroepidemiological studies show that over 50% of adults have been exposed to this pathogen at some time during their life. In addition, seroprevalence increases with age due to e.g. recurring infections or reactivation of chronic infections. In industrialized countries, the most prominent increase in seroprevalence is seen during the years 5 to 20, and in the elderly population seroprevalence is up to around 75%. (Saikku 1992, Kuo et al. 1995b.) In a study with children in Finland, it has been shown by an enzymeimmunoassay (EIA) method that primary *C. pneumoniae* infections occur at early ages (0.6–1.1 years), but that the IgG antibodies caused by these early infections usually decline rapidly; whereas an IgA response was seldom detected. Seroprevalence increased clearly at school age; IgG responses were more persistent and IgA antibodies were produced more often. No difference in IgG and IgA prevalence was seen between boys and girls. (Volanen et al. 2003.) In the adult population, seroprevalence is higher among men than among women.
Acute infections

*C. pneumoniae* is a respiratory pathogen that causes upper and lower respiratory tract infections. *C. pneumoniae* is thought to cause ~10% of acute pneumonia cases that are often mild with prolonged onset, and ~5% of acute bronchitis and sinusitis cases. Pharyngitis is frequently associated with *C. pneumoniae* infection and is often relatively severe. In addition, common colds are frequent in *C. pneumoniae* infections or the infections may also be asymptomatic. Epidemics have been reported in all seasons of the year, and their course is usually long and the spread of infection from case to case is relatively inefficient (Ekman et al. 1993a). Reinfections are thought to be common especially in elderly people, and among them fewer can be severe and last for long periods. Generally, reinfections can, however, be either milder or more severe than primary infections. (Grayston et al. 1990, Saikku 1992.) Coinfections, especially with *Streptococcus pneumoniae* and *Mycoplasma pneumoniae*, may occur (Kauppinen et al. 1995, Monno et al. 2002).

In vitro persistent infections

In the case of chlamydiae the term “persistence” refers to a situation where a long-term association between them and their host cell is established; this situation is characterized by chlamydiae remaining viable but culture-negative (Beatty et al. 1994b). At the organism level, persistent infection means the immune system of the host does not eliminate the pathogen but it remains inside the host and may over time continue to cause damage. In the case of chlamydiae, this persistence may last from several months to years, and often without any obvious illness as an outcome.

In vitro persistent infection, characterized by an altered developmental cycle and development of aberrant forms, can be triggered by IFN-γ, nutrient depletions, and antibiotics (Beatty et al. 1994b, Hogan et al. 2004). Treatment of host cells with medium levels of IFN-γ during infection results in large RBs with abnormal metabolism (Shemer & Sarov 1985, Beatty et al. 1993, Pantoja et al. 2001). IFN-γ induces indoleamine 2,3-dioxygenase, which catalyzes the degradation of tryptophan (Byrne et al. 1986, Beatty et al. 1994a). In addition, it limits
chlamydial growth by nitric oxide (NO) induction, and intracellular iron depletion (Igietsene et al. 1998). The effect of IFN-γ treatment is reversible upon removal of IFN-γ (Beatty et al. 1993), and as a result internal reorganization of the enlarged forms and emergence of morphologically normal RBs and EBs is seen (Beatty et al. 1995).

Depletion of amino acids or glucose in a culture medium has been described to cause persistent forms of *C. trachomatis* in McCoy cells. Interestingly, amino acid concentrations seen in blood were found to induce aberrant forms. However the minimum requirement of each amino acid by chlamydia, rather than the total amino acid concentration within cells, may be the limiting factor in this case (Harper et al. 2000).

Iron participates in important cell functions like electron transport and DNA synthesis, and it is likely that chlamydiae transport and use iron from their host cells. Depletion of iron by treatment with deferoxamine mesylate (DAM), which removes free cellular iron, has been reported to cause persistent forms of *C. pneumoniae* in a cell culture, and this inhibition has been shown to be reversible by addition of iron-saturated transferrin. This suggests that iron levels may affect the outcome of chlamydial infections also *in vivo* (Al-Younes et al. 2001).

Treatment of infected cells with antibiotics may result in persistent forms of chlamydia, with the type and amount of antibiotics, and the time of the developmental cycle during treatment determining the primary molecular target. Mainly, treatment that targets bacterial protein or RNA synthesis results in inhibition of differentiation either from EBs to RBs or from RBs to EBs, and treatment that targets DNA or peptidoglycan synthesis prevents RB-to-EB differentiation. (Beatty et al. 1994b, Hogan et al. 2004.) Subinhibitory concentrations of antibiotics used for treatment of *C. pneumoniae* infection have also been shown to induce persistence in HeLa cells (Gieffers et al. 2004).

Exposure to tobacco smoke has been shown to induce persistent chlamydial infection in human laryngeal epidermoid carcinoma cells (Hep-2) (Wiedeman et al. 2004) and in human aortic and coronary artery endothelial cells. In the latter study, reactivation of chlamydia was seen after removal of the exposed medium and addition of a L-tryptophan containing medium (Wiedeman et al. 2005).

Spontaneous appearance of persistent forms has also been described in a continuous *C. pneumoniae* culture in Hep-2 cells where no new chlamydia is added and no centrifugation and cycloheximide are used. The number of
persistent forms in this model is small, with most infected host cells showing normal productive infection. (Kutlin et al. 2001.)

**In vivo persistent infections**

It is well known that chlamydial species have a tendency to cause persistent infections *in vivo*. In the case of *C. trachomatis*, persistent infections have been associated with trachoma and tubal factor subfertility (den Hartog et al. 2006, Wright et al. 2008). Persistence of *C. pneumoniae* pulmonary infection has been described in Swiss Webster mice, where viable *C. pneumoniae* can be isolated up to 42 days after infection and lung pathology may remain visible up to 60 days (Yang et al. 1995). In humans, persistent pulmonary *C. pneumoniae* infections and cases where extended antibiotic treatment has only caused temporary improvement have been described (Falck et al. 1994, Falck et al. 1996, Miyashita et al. 2002). In addition, persistent *C. pneumoniae* infection has been associated with several different diseases. These diseases themselves are also often chronic in nature, and include e.g. cardiovascular diseases like coronary heart disease, acute myocardial infarction, stroke, transient ischemic attack, and abdominal aortic aneurysm; neurological diseases like multiple sclerosis and Alzheimer’s disease; respiratory tract diseases like asthma and chronic obstructive pulmonary disease (COPD); and other disease like lung cancer, non-Hodgkin lymphoma, and reactive arthritis (Saikku 1999, Saikku 2002).

**Molecular differences in acute and persistent infections**

Genetic differences between *Chlamydia* species and strains in genes that may play a role in the development of persistent infections have been described. *C. pneumoniae* and several serovars of *C. trachomatis* are relatively more sensitive to IFN-γ treatment than are *C. psittaci* and *C. muridarum*. This may be due to variation in the *lifA* gene that encodes for a toxin capable of blocking IFN-γ production. In addition, differences in genes of the tryptophan biosynthesis pathway may play a role. *C. psittaci* has the most complete set of these genes, whereas the genome of *C. pneumoniae* lacks both the *lifA* and tryptophan pathway genes. (Xie et al. 2002.) On the other hand, differences between the pulmonary and vascular strains of *C. pneumoniae* in copy numbers of *tyrP* gene, which encodes for tyrosine and tryptophan permease, have been reported. Only one copy was observed in the vascular strains, whereas most of the pulmonary
strains had multiple copies of this gene. In addition, the multiple copy strains showed a higher transcription of \textit{tyrP} and higher tyrosine uptake than did the single-copy strains; however, no difference in growth of the strains under tyrosine- and tryptophan-limiting conditions was seen. The authors suggested that due to the \textit{tyrP} polymorphism, there may be differences in the ability of \textit{C. pneumoniae} to enter persistent growth, which could affect their ability to survive in monocytes and vascular wall cells. (Gieffers \textit{et al.} 2003.)

Gene expression studies have shown several differences between acute and persistent infections; the differentially expressed genes encode proteins that function e.g. in stress response, ribosome function, carbohydrate metabolism, structure, regulation of RB-EB differentiation, and T3S (Mathews \textit{et al.} 2001, Polkinghorne \textit{et al.} 2006). On the host cell side, gene expression studies have also identified several genes with altered expression during induced persistent \textit{C. pneumoniae} infection; these genes function e.g. in immunity and inflammation, adhesion, and cell growth and proliferation (Mannonen \textit{et al.} 2007). During longer periods (≥4 days) of \textit{in vitro} persistence in HeLa cells, IFN-γ- and penicillin-induced persistence have been reported to cause silencing of host cell gene expression (genes known to be up-regulated in acute infection). Whereas, in an iron depletion model long-lasting gene activation was seen, suggesting that also \textit{in vivo} there may be different cell responses in persistent infections (Peters \textit{et al.} 2005).

\textit{Detection methods}

As a response to several different detection methods, cut-off values, and variable positivity rates seen between different studies (Dowell \textit{et al.} 2001), in 2001 the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada) published recommendations for detecting \textit{C. pneumoniae} infection.

Direct isolation of \textit{C. pneumoniae} by culture is relatively difficult. As an obligate intracellular bacterium it can only be grown in an eukaryotic cell culture, preferably Hep-2 or HL cells (or in the yolk sac of embryonated chicken eggs) (Grayston \textit{et al.} 1986, Cles & Stamm 1990). Usually bacteria are inoculated into host cells with centrifugation and after passage, inclusions in cell monolayers are detected with fluorescently labeled antibodies and microscopic examination. For respiratory specimens, two passages in addition to primary isolation and for tissue samples, 4–6 additional passages have been recommended. Problems associated
with culture include poor growth of \emph{C. pneumoniae}, contamination with \emph{Mycoplasma} species, technical complexity, and well-to-well contaminations, especially when microtiter plates are used. The reliability of cell culture results also depends on the types of specimens and their treatment, culture medium, cell type, and the overall procedure used (Dowell \textit{et al.} 2001). Successful culture of \emph{C. pneumoniae} from atherosclerotic tissue samples has only been reported by a few studies (Ramirez 1996, Jackson \textit{et al.} 1997, Maass \textit{et al.} 1998a, Cuffini \textit{et al.} 2006). The reason for this may be that in these samples the organism is in a persistent form that is considered culture-negative. However, a recent study reported quite frequent (24.8\%) isolation of infectious \emph{C. pneumoniae} from white blood cells of blood donors using a mouse monocyte macrophage cell line (Cirino \textit{et al.} 2006).

A MIF test (Wang 2000, Paldanius \textit{et al.} 2003) with purified EBs as an antigen is considered the method of choice for detection of acute \emph{C. pneumoniae} infection (Dowell \textit{et al.} 2001). In a MIF test, a fourfold rise in serum \emph{C. pneumoniae} IgG between paired serum samples obtained 4–8 weeks apart or an IgM titre $\geq 16$ can be considered a marker of acute infection. An IgG titre $\geq 16$ is thought to mark past or persistent infection and IgG $\geq 512$ may mark acute infection, but using a single measurement of IgG is not considered a good method for determining acute infection. (Wang 2000, Dowell \textit{et al.} 2001.) Especially in older patients, the presence of a rheumatoid factor (IgM-class autoantibody reactive with the Fc region of IgG) in serum may cause a false positive IgM result; this can be avoided by adsorbing IgG from the serum prior to testing for IgM (Verkooyen \textit{et al.} 1992, Wang 2000). This treatment is favorable also in IgA titrations (Jauhiainen \textit{et al.} 1994). Differences in MIF methods and results between different laboratories have been reported (Peeling \textit{et al.} 2000, Littman \textit{et al.} 2004). Clearly, drawbacks of MIF are lack of standardized reagents, technical complexity, and subjective end points. However when performed properly, MIF is a sensitive and specific method for detecting acute infections (Peeling \textit{et al.} 2000). Chronically elevated (elevated in repeated samples) antibodies against \emph{C. pneumoniae} can be considered a marker of persistent infection; this, however, requires several serum samples taken over time and is not possible in all studies. Since the biological half-life of IgA antibodies is usually shorter (only 5–6 days) than that of IgG antibodies, it has been proposed that the presence of IgA antibodies might better represent persistent infection (Saikku \textit{et al.} 1992, Saikku 1999, Falck \textit{et al.} 2002). However, the meaning of antibody positivity in relation to persistent infections still remains unclear.
The other serological methods for detecting *C. pneumoniae* infection include complement fixation (CF), whole inclusion fluorescence, and commercial EIA methods. However, use of these methods has not been recommended due to several drawbacks associated with each of these methods. The CF test is cross-reactive with other *Chlamydia* and possibly with enteric bacteria species, its sensitivity is low in reinfections, and the reagents are not easily available. Whole inclusion fluorescence tests lack species specificity and commercial EIAs have been reported to have problems with sensitivity and specificity. (Ekman et al. 1993b, Dowell et al. 2001.) On the other hand, EIA tests are promising since they are often commercially available, easy to perform, their end point is objective, and the results can be read electronically. An LBP-based method for detecting cLPS from serum samples was recently published (Tiirola et al. 2006). The advantage of this method is that it has potential use in detecting persistent infections, whereas the problem is that it cannot differentiate between the different chlamydial species.

PCR has been used to detect *C. pneumoniae* from different sample types including respiratory samples, peripheral blood monocytes, serum, and vascular tissue. Several different methods that include conventional, hot-start, touch-down, nested, and real-time techniques have been published, but no standardized methods exist. In addition, collection and processing of samples and use of quality controls differ between studies and laboratories. Indeed, great variability, from 0% up to 100%, in positivity rates is evident in the literature (Juvonen et al. 1997, Daus et al. 1998, Jantos et al. 1999, Ciervo et al. 2003, Mygind et al. 2003, Kozarov et al. 2006). Significant variation between different PCR assays performed on the same samples in one laboratory has also been reported; of eight positive samples only two were reported to be positive by more than one of the PCR methods tested (Apfalter et al. 2003). In addition, multicenter comparisons have shown interlaboratory variation in PCR positivity of the same sample sets consisting of different sample types (Apfalter et al. 2001, Chernsky et al. 2002, Loens et al. 2006), and one study suggested that the positive results were likely to originate from contamination(s) (Apfalter et al. 2002). On the other hand, a low concentration and patchy distribution of *C. pneumoniae* DNA in atherosclerotic tissue may play a role in observed positivity rate differences. It has been reported that if only one 30-μm-thick section is studied, the chance of a true positive finding is ~35–42% and that to obtain a 95% chance at least 15 sections need to be studied (Cochrane et al. 2003). In respiratory samples, *C. pneumoniae* DNA has been found by PCR in both upper- and lower respiratory tract samples. In
some cases a higher PCR positivity rate has been reported for the upper respiratory tract. However, it is unclear to what extent this reflects carriage versus invasive disease. (Murdoch 2003.)

### 2.1.6 Treatment

To treat acute respiratory *C. pneumoniae* infections, antibiotics like macrolides (erythromycin, roxithromycin, clarithromycin, and azithromycin), fluoroquinolones (the newer ones like levofloxacin, moxifloxacin, gemifloxacin, and garenoxacin being more efficient), or tetracyclines can be used, and intensive, prolonged therapy is recommended (Blasi 2004). However, it has been shown in a continuous cell culture model that 30-day treatment with azithromycin, clarithromycin, or levofloxacin with concentrations similar to those achieved in epithelial lining fluid reduced but did not eliminate *C. pneumoniae* (Kutlin et al. 2002). In a study that focused on eradication of replicating *C. pneumoniae* from coronary endothelial cells and smooth muscle cells, all the antibiotics tested were found to be effective, with rifampin showing the best results. Macrolide roxithromycin, and quinolone moxifloxacin were the most efficient drugs (Gieffers et al. 2001b). However, rifampin and azithromycin were not able to eradicate *C. pneumoniae* from cultured human monocytes, but instead a persistent infection was developed (Gieffers et al. 2001a).

Vaccination against *C. pneumoniae* infections could help prevent not only respiratory diseases caused by the pathogen but, if efficient against persistent infection, possibly also cardiovascular diseases. However, no such vaccine is available to date. The design of vaccines against *C. pneumoniae* infection is complicated by exacerbated immunopathology, possibly due to immune response against some chlamydial components. Thus, vaccine research has mainly focused on subunit vaccines and DNA vaccines against e.g. MOMP, Omp2, and Hsp60. (Puolakkainen & Mäkelä 1999.) Some new vaccine candidates have been proposed in recent studies. In a genome-wide screening of multiple vaccine candidates, 53 recombinant proteins that induced mouse *C. pneumoniae*-binding antibodies were identified, and in further studies six of these were reported to induce *in vitro* neutralizing antibodies and four (Pmp2, Pmp10, OmpH-like, and enolase) also inhibited dissemination of *C. pneumoniae* in a hamster model (Finco et al. 2005). Vaccination with LcrE (a putative lid of the type III secretion system) fusion protein was recently found to induce CD4+ and CD8+ T cell activation,
type 1 cytokine response, and neutralizing antibodies, and to be effective in eliminating *C. pneumoniae* infection in mice (Thorpe *et al.* 2007).

### 2.2 Recognition of and responses to *C. pneumoniae* by the human immune system

#### 2.2.1 Innate immunity

The mechanisms of innate immunity exist prior to an encounter with microbes and are rapidly activated upon contact with infectious agents. Components of innate immunity include epithelial barriers (skin, lining of the gastrointestinal and respiratory tracts), neutrophils, mononuclear phagocytes, natural killer (NK) cells, and several plasma proteins. Through pattern recognition receptors, innate immunity recognizes conserved microbial structures like double-stranded RNA, unmethylated CpG DNA sequences, proteins, and complex lipids and carbohydrates that are common in microbial pathogens but are not present in mammalian cells. Cytokines secreted especially by macrophages and NK cells activate phagocytes and induce inflammation, a cellular reaction of innate immunity that consists of recruitment and activation of leukocytes and upregulation of several plasma proteins. The acute symptoms of chlamydial infections are a result of acute inflammatory reaction at the primary infection site. If the host’s immune response is not able to eradicate infection, it may persist together with inflammation and cause tissue damage at the site of infection. Perhaps the best-established examples of this situation are blinding trachoma and tubal factor infertility caused by *C. trachomatis* infections. (Kuo 1999.)

**Neutrophils and macrophages**

The likely route of entry for *C. pneumoniae* is through the airway epithelium. *C. pneumoniae* can infect epithelial cells, promote activation of the NF-κβ pathway, and induce production of adhesion molecules and chemokines like intercellular adhesion molecule 1 (ICAM-1) and IL-8 in them. Infection of epithelial cells also induces transepithelial migration of polymorphonuclear neutrophils. (Jahn *et al.* 2000.) Leukocytes are recruited to the site of infection by adhesion molecules and chemokines expressed by epithelial cells. Neutrophils and monocytes are the first leukocytes that arrive to the infection site. In acute
Chlamydial infections the cellular infiltrates are dominated by neutrophils, whereas in persistent infections mononuclear leukocytes consisting mainly of lymphocytes have been reported to be dominant; the infiltrates also contain plasma cells, eosinophils, and macrophages (Kuo 1999). Neutrophils are the most abundant circulating white blood cells and contain granules and lysosomes that are filled with enzymes like lysozyme, collagenase, and elastase, and other microbicidal substances. Neutrophils can ingest chlamydial EBs; however *C. pneumoniae* is capable of infecting them and delaying their spontaneous apoptosis (van Zandbergen et al. 2004). Monocytes and macrophages are important mediators of innate and adaptive immune responses. Macrophages persist longer than neutrophils in the site of inflammation, and thus are usually the dominant effector cell type seen in the later stages of the innate immune response. *C. pneumoniae* is also capable of infecting monocytes and macrophages, and it is thought to disseminate from the lungs to other parts of the body mainly inside monocytes (Gaydos et al. 1996, Moazed et al. 1998).

Neutrophils and monocytes/macrophages recognize and ingest microbes through several different receptors. Mannose receptors bind terminal mannose and fucose residues typically found in microbial cell walls, scavenger receptors bind modified LDL but also a variety of microbes, and opsonin receptors like high-affinity Fcγ receptors (FcγRI) and the type 1 complement receptors (CR1) recognize and promote phagocytosis of microbial structures that have been coated with opsonins (antibodies, complement proteins, mannose-binding lectin, fibronectin, fibrinogen, and CRP). After ingestion, microbes end up in phagosomes that usually fuse with lysosomes, leading to destruction of the microbe by reactive oxygen intermediates (ROIs), reactive nitrogen intermediates, and proteolytic enzymes. In addition, proteins are processed for presentation to CD4+ T lymphocytes by class II major histocompatibility complex (MHC) pathway. Chlamydiae however, are able to prevent fusion of phagosomes with lysosomes (Friis 1972, Eissenberg et al. 1983). G protein-coupled receptors recognize microbes and some mediators produced as a response to infection, and mainly stimulate migration of leukocytes to the site of infection. TLRs again activate phagocytes to respond to different components of microbes.

**Natural killer cells**

The main function of natural killer (NK) cells is to recognize and kill cells infected by viruses and some other intracellular microbes. Activation of NK cells
is regulated by a balance of inhibiting and activating signals received from receptors belonging to several different families. The activating receptors recognize ligands that are expressed on the surface of infected or stressed cells and the inhibiting receptors bind to class I MHC molecules. If the inhibitory receptors are engaged, no activation of NK cells occurs. In infected host cells, expression of class I MHC molecules is often inhibited. In addition, NK cells can recognize and destroy antibody-coated targets. Activated macrophages secrete IL-12, which stimulates IFN-γ production and cytolytic activity of NK cells. NK cells kill their target cell by releasing perforin, which creates pores in the target cell membranes and granzymes that induce apoptosis of the target cells. By secreting IFN-γ, NK cells also activate macrophages to more efficiently kill phagocytosed bacteria. The role of NK cells in C. pneumoniae infection is not clear. Based on studies with mice, it has been suggested that NK cells do not play a major role in secretion of protective levels of IFN-γ after C. pneumoniae infection (Rottenberg et al. 2000, Rothfuchs et al. 2004a).

**Dendritic cells**

Most dendritic cells (DCs) are myeloid DCs that are related to mononuclear phagocytes. Immature DCs are found e.g. in epithelia of the skin, and the gastrointestinal and respiratory tracts. These professional antigen-presenting cells (APCs) bind and ingest microbes and microbe-infected cells (cross-presentation), and transport the antigens to be presented in lymph nodes, where the DCs very efficiently stimulate naïve T cells. Antigens may also arrive to lymph nodes in a soluble form, where they can be extracted by DCs, macrophages, or B cells and presented to naïve or effector T cells. DCs present antigens in both class II and class I MHC molecules and can thus activate both CD4+ helper T cells and CD8+ cytolytic T cells. Recognition of C. pneumoniae by DCs is thought to be mainly Toll-like receptor 2 (TLR-2)-dependent, and it induces activation of NF-κB, secretion of IL-12 and TNF-α, and increases expression of MHC II and costimulatory molecules. (Prebeck et al. 2001, Costa et al. 2002.) It has been shown that DCs pulsed with heat-inactivated chlamydia activate and induce Th1 type CD4+ response and protective immunity (Shaw et al. 2001). C. trachomatis-infected DCs process and present chlamydial antigens but fail to stimulate proliferation of naïve CD8+ T cells, or after adoptive transfer, generation of antigen-specific CD8+ T cells. This may be due to insufficient presentation of chlamydial antigens. Thus, cross-presentation of antigens during chlamydial
infection may be important in activation of CD8+ T cells. (Steele et al. 2004.) C. pneumoniae has also been reported to be capable of infecting and surviving in DCs, which promotes TNF-α production (Wittkop et al. 2006).

Pattern recognition receptors

TLRs are expressed by a variety of different cell types and recognize different conserved microbial structures. Upon contact with their target, they activate innate immunity responses. Currently, 10 members of the TLR family have been identified in humans. The TLRs that are currently thought to be most important in recognizing C. pneumoniae infection are TLR-2 and TLR-4. The inflammatory responses mediated by TLR-2 and TLR-4 use a MyD88-dependent pathway, and signaling through them causes NF-κB activation (Takeda et al. 2003, Pålsson-McDermott & O’Neill 2004).

TLR-4 is a receptor for e.g. LPS (Poltorak et al. 1998, Qureshi et al. 1999, Vogel & Fenton 2003), respiratory syncytial virus fusion protein (Kurt-Jones et al. 2000), cHsp60 (Bulut et al. 2002), and possibly some endogenous ligands like human Hsp60 (hHsp60) and fibronectin (Ohashi et al. 2000, Okamura et al. 2001, Tsan & Gao 2004). For efficient LPS signaling, some additional factors are needed. In blood, LPS is bound by an acute phase protein called LPS-binding protein (LBP) (Tobias et al. 1986), and LPS-LBP complexes are then transferred to CD14. Two forms of CD14 exist: soluble CD14 (sCD14), found in plasma, and membrane-bound CD14 (mCD14), expressed e.g. by cells of the monocyte/macrophage lineage (Antal-Szalmás 2000). The transfer of LPS from LPS-LBP complexes to mCD14 causes interaction between mCD14, the LPS receptor TLR-4, and MD-2. This leads to NF-κB activation and production of proinflammatory cytokines (Raetz et al. 1991, Heumann & Roger 2002, Pålsson-McDermott & O’Neill 2004). LBP also catalyzes formation of complexes between LPS and sCD14. sCD14 has two functions: sCD14 complexed with LPS can activate cells that themselves do not express CD14 (Pugin et al. 1993), whereas in high concentrations it may inhibit binding of LPS-LBP complexes to mCD14 by competing over LPS (Grunwald et al. 1993) and also increase the release rate of LPS from the cell-surface thereby diminishing cellular activation (Schutt et al. 1992). Moreover, both LBP and sCD14 also transfer LPS to plasma lipoproteins like high-density lipoprotein (HDL) and thus promote LPS neutralization/removal (Ulevitch et al. 1979, Wurfel et al. 1995, Kitchens et al. 1999, Kitchens et al. 2001). It has recently been shown that CD14 also
participates in TLR-2 signaling by acting as a membrane receptor for e.g. peptidoglycan and lipomannan and by presenting them to TLR-2 (Schröder et al. 2003, Vignal et al. 2003).

TLR-2 is a receptor for e.g. peptidoglycan, lipoproteins/lipopeptides, and lipoteichoic acid (Hirschfeld et al. 2001, Takeda et al. 2003). It has also been reported that unlike most LPSs, cLPS could be recognized through TLR-2 rather than TLR-4 (Erridge et al. 2004). However, this result may have been due to contamination of the cLPS preparation with chlamydial proteins. Highly purified cLPS has been shown to induce cell activation via TLR-4/MD2 and to be strictly CD14-dependent (Heine et al. 2003). On the other hand, recognition of chlamydial Hsp60 has been reported to happen through both TLR-4 and TLR-2, and in this case TLR-2 may play a more prominent role. It has also been suggested that cHsp60 may be an important component for C. pneumoniae-induced cell activation (Prebeck et al. 2001, Costa et al. 2002, Netea et al. 2002). In addition, C. pneumoniae-induced foam cell formation has been suggested to be mainly TLR-2-dependent (Cao et al. 2007).

Recently identified nucleotide-binding oligomerization domain (Nod) proteins have been implicated to function in intracellular pattern recognition. Only a few members of this family have been characterized so far. Nod1 has been reported to mediate NF-κB activation that has been induced by peptidoglycans containing meso-diaminopimelate acid, which is mainly found in Gram-negative bacteria. (Girardin et al. 2003.) It has recently been shown that intracellular C. pneumoniae (viable or heat-inactivated) activates NF-κB through Nod1, and that Nod1 is crucial for chlamydia-induced IL-8 production in endothelial cells (Opitz et al. 2005). It has also been reported that chlamydia can induce IFN-γ production in macrophages both by TLR-4-dependent and by TLR4-independent pathways, and that these pathways are complementary to each other (Rothfuchs et al. 2004b). It may thus be that Nod proteins play an important role in C. pneumoniae-induced cell activation.

**Acute phase reactants**

LPS signaling through TLR-4 induces production of proinflammatory cytokines like interleukin 6 (IL-6), IL-1, and TNF-α, which promote synthesis of acute-phase reactants in liver. IL-6 induces production of CRP, fibrinogen, LBP (Grube et al. 1994, Wan et al. 1995), and possibly sCD14 (Bas et al. 2004), whereas IL-1 and TNF-α mainly induce synthesis of serum amyloid A (SAA). CRP typically
binds bacterial phospholipids and functions as an opsonin for bacteria and circulating immunocomplexes, and may contribute to complement activation and clearance of cellular debris. During acute phase reactions, the serum level of CRP may increase by 1000-fold and even slightly elevated levels may predict future cardiovascular events (Yeh & Willerson 2003).

In disseminated infections, cytokines induced by LPS may cause systemic inflammatory response syndrome (SIRS), which in mildly affected patients consists of neutrophilia, fever, and elevated serum acute-phase reactants. When severe infections lead to the presence of bacteria and LPS in blood (sepsis), high levels of cytokines (e.g. TNF-α) are produced as a response to LPS. This may result in disseminated intravascular coagulation (DIC), damage to the lungs (adult respiratory distress syndrome, ARDS) and liver (hypoglycemia), in particular, and heart failure and loss of perfusion pressure. The combination of DIC, hypoglycemia, and heart failure is called septic shock, a condition that is often fatal.

**Complement system and mannose-binding lectin**

The complement system is composed of several plasma proteins that are activated upon contact with microbes. The complement can be activated by antibodies bound to their target surface (classical pathway), by direct recognition of some microbial surface structures (alternative pathway), or by mannose-binding lectin (MBL) bound to microbes (lectin pathway). If the complement cascade proceeds to its end, it results in formation of a membrane attack complex and lysis of the microbe. In addition, it promotes inflammation and functions as an opsonin. MBL binds carbohydrates with terminal mannose and fucose of microbial glycoproteins and glycolipids. MBL also mediates phagocytosis of the particles it has opsonised. It has been shown that *C. trachomatis* activates the complement cascade, resulting in chemotaxis of neutrophils (Megran et al. 1985), and that early complement components may enhance neutralization of chlamydial infectivity (Lin et al. 1992). MBL has been shown to inhibit infectivity of *C. trachomatis* and to bind it, possibly through MOMP (Swanson et al. 1998).

**2.2.2 Adaptive immunity**

Immune responses of adaptive immunity are stimulated by exposure to infectious agents. Since its receptors are produced by somatic recombination of gene
segments, adaptive immunity is capable of recognizing a large number of microbial and nonmicrobial substances, and it can differentiate between even closely related molecules. The magnitude and defensive capabilities of adaptive immune responses increase with each successive exposure to a particular microbe. Components of adaptive immunity include lymphocytes and antibodies secreted by B lymphocytes. B lymphocytes and antibodies are components of humoral immunity and T cells are components of cell-mediated immunity.

B lymphocytes

Activation and differentiation of naïve B lymphocytes to antibody producing cells is triggered by antigen-induced clustering of their membrane immunoglobulins. The antigen is also internalized by B lymphocytes, and if it is a protein it is presented in class II MHC for helper T cells that stimulate clonal expansion, isotype switching, affinity maturation, and differentiation to memory B cells. However, many important nonprotein antigens also induce antibody production through mechanisms that do not involve helper T cells. Activated B lymphocytes produce and secrete immunoglobulins (Igs) of various isotypes. Some of the antibody-producing cells migrate to bone marrow and may survive there for years after the original infection, producing low levels of antibodies. Upon subsequent exposures to the same antigen, the antibodies produced by long-living B cells provide initial protection and the memory cells are rapidly activated to produce more antibodies. The functions of antibodies are to neutralize microbes and toxins, to opsonise microbes and thus promote their phagocytosis, to induce antibody-dependent cell-mediated cytotoxicity, and to activate the complement system. C. pneumoniae infections induce production of antibodies against several different antigens (Campbell et al. 1990b, Essig et al. 1999, Campbell et al. 2001, Penttilä et al. 2006). However, it is likely that antibody response against C. pneumoniae is not protective in humans, since reinfections are common, and as an intracellular pathogen, chlamydia may escape humoral responses (Puolakkainen & Mäkelä 1999). Studies with mice have suggested that antibodies may reduce the amount of infective EBs but do not completely clear the infection or protect against inflammatory changes (Kaukoranta-Tolvanen et al. 1995). However, increasing IgA antibody content in local secretions has been suggested to correlate with diminishing C. pneumoniae counts in mouse lungs (Penttilä et al. 2006).
Both naïve CD4+ and CD8+ T cells are activated in peripheral lymphoid organs by DCs that present antigens that the T cells recognize, and by costimulators and cytokines also provided by the APCs. Antigen-stimulated T cells differentiate to effector and memory T cells that can also recognize antigens presented to them in nonlymphoid tissues and by macrophages or B cells. CD4+ T cells may differentiate to Th1 cells that activate macrophages, neutrophils, and IgG production, or to Th2 cells that stimulate production of IgE, activate eosinophiles, and inhibit macrophage activation. In humans, these lineages are most clearly observed in chronic immune reactions. The cell lineages also secrete different cytokines, with IFN-γ being the signature cytokine of Th1 cells and IL-4 and IL-5 those of Th2 cells. CD8+ T cells differentiate to functional cytolytic T cells that recognize and destroy infected host cells in a manner similar to that of NK cells. Cell-mediated immunity is important in protection against *C. pneumoniae*, and CD8+ T cells, in particular, play an important role in both primary infections and reinfections (Penttilä *et al.* 1999, Rottenberg *et al.* 1999). As a response to IL-12 secreted by activated macrophages, T cells (in addition to NK cells and macrophages) secrete IFN-γ that is crucial for control of chlamydial growth, but may also be a triggering factor for formation of persistent forms of chlamydia (Rottenberg *et al.* 2002). The three mechanisms triggered in cells by IFN-γ include depletion of tryptophan, induction of NO, and depletion of iron, and they seem to work together in intracellular responses against chlamydia (Igietseme *et al.* 1998, Rottenberg *et al.* 2002). Several epitopes from *C. pneumoniae* proteins like Hsp70, Omp2, and MOMP have been reported to be recognized by CD8+ cells in mice (Sarén *et al.* 2002, Wizel *et al.* 2002). *C. pneumoniae* reactive T cells have also been isolated from human carotid artery plaques; many of these T cells were reactive to cHsp60 (Morin *et al.* 2000). Chlamydiae may escape from T cell responses by inducing IL-10 production that down-regulates expression of class I MHC molecules (Caspar-Bauguil *et al.* 2000) and by secreting a chlamydial protease CPAF that degrades a host cell transcription factor, RFX5, needed for MHC expression (Dong *et al.* 2005).
2.3 Human gene polymorphisms in infection susceptibility, inflammatory and innate immunity reactions, and CVD

Several studies have addressed the association of genetic variation in genes important in innate immunity and inflammation with susceptibility to sepsis and infections, but also with chronic inflammatory diseases like CVD, type II diabetes, and rheumatoid arthritis. In addition, the possible mechanisms of these associations have been studied by comparing genetic variation and variation in the serum or expression levels of several marker molecules. In many cases there have also been contradicting reports about functionality, and many of the associations found have not been confirmed by other study groups. This may be due to false positive or false negative findings, or differences in associations between different populations. The reasons for false positive or negative reports include e.g. associations created by change due to not correcting for multiple testing, underpowered sample size, missing data, genotyping errors, differences or problems in defining the phenotype, genetic or environmental heterogeneity of the study population, and gene-gene and/or gene-environment interactions. (Newton-Cheh & Hirschhorn 2005, Lunetta 2008, Zintzaras & Lau 2008.) The following sections focus on gene polymorphisms that have been reported to be functional and possibly associate with several different diseases. These gene polymorphisms could also play a role in susceptibility to developing persistent C. pneumoniae infection and/or in modifying the effect of C. pneumoniae on the pathogenesis of several chronic diseases.

2.3.1 LBP

In the LBP gene there is a single-nucleotide polymorphism (SNP) C\textsubscript{1341}$\rightarrow$T that causes a Leu\textsubscript{436}$\rightarrow$Phe change (SNP accession no. rs2232618) (Hubacek \textit{et al.} 2001). In the original publication the polymorphism was reported as an C\textsubscript{1306}$\rightarrow$T (Pro\textsubscript{436}$\rightarrow$Leu) change (Hubacek \textit{et al.} 2001), but based on the GeneBank sequences (GeneBank accession nos. AL080249, NM004139, and NP004130), the amino acid number 436 in the LBP gene is a phenylalanine, not a proline. The codon (TTC) for amino acid number 436 is located between the bases 1341 and 1343, calculated from the beginning of the mRNA sequence (NM004139), and there is a recorded C$\rightarrow$T change in the nucleotide number 1341 that causes a change of leucine to phenylalanine (SNP accession no. rs2232618). The PCR primers used in Hubacek \textit{et al.} (Hubacek \textit{et al.} 2001) are located near this
polymorphism, and the authors confirmed that this is the polymorphism reported by them as a C\textsubscript{1306}$\rightarrow$T (Pro\textsubscript{436}$\rightarrow$Leu) change, and that the differences observed were caused by a change in the sequence data since the year 2001 (personal communication). The same original article also reported the presence of a T\textsubscript{292}$\rightarrow$G (Cys\textsubscript{98}$\rightarrow$Gly) change in the \textit{LBP} gene, the G allele of which was associated with sepsis in male patients (Hubacek \textit{et al.} 2001). However, the existence of this polymorphism was not confirmed by another study group, instead they reported a finding of a T\textsubscript{291}$\rightarrow$C polymorphism that was not associated with sepsis (Barber & O’Keefe 2003). Hubacek \textit{et al.} also reported a weaker (statistically non-significant) association between the C\textsubscript{1341} (Leu\textsubscript{436}) allele and sepsis in males. Persons homozygous for the G\textsubscript{292} and/or C\textsubscript{1341} alleles also tended to be sepsis non-survivors, but the frequency of these homozygous individuals was low, and no statistical analysis was performed (Hubacek \textit{et al.} 2001). In another study, individuals homozygous for the C\textsubscript{1341} (Leu\textsubscript{436}) allele were about three times more frequent among the myocardial infarction patients than among the controls (2.3% and 0.7%, respectively), but no statistically significant association between the polymorphism and myocardial infarction was found (Hubacek \textit{et al.} 2002). The chromosomal region around the \textit{LBP} gene has been reported to show an association with type II diabetes and for four SNPs (rs2232578, rs1739654, rs2232592, and rs1780627) in the \textit{LBP} gene, a significant association was reported (Takeuchi \textit{et al.} 2007). Chien \textit{et al.} (2008) recently reported an association between an \textit{LBP} gene promoter polymorphism (SNP 1683, rs2232571) and serum LPB concentration. In addition, the same promoter variant was associated with a fivefold higher risk of death due to Gram-negative bacteremia after transplantation.

\textbf{2.3.2 CD14}

There is a C\textsubscript{−260}$\rightarrow$T polymorphism (rs2569190) in the promoter region of the \textit{CD14} gene. In the literature this polymorphism is also referred to as C\textsubscript{−159}$\rightarrow$T depending on whether the translation or the transcription start site is considered as site 0. The T allele and TT genotype may be connected to higher expression of CD14 (Baldini \textit{et al.} 1999, Hubacek \textit{et al.} 1999, Ito \textit{et al.} 2000, Giacconi \textit{et al.} 2007). It was recently reported that in whole blood cultures, the T allele is associated with higher CD14 mRNA expression, and with higher sCD14, IL-6, and TNF-\textalpha, but lower IL-10 production (Lin \textit{et al.} 2007). Association of the polymorphism with several different diseases has been suggested, e.g. the T allele
and TT genotype may be associated with a higher risk of myocardial infarction (Hubacek et al. 1999, Unkelbach et al. 1999, Shimada et al. 2000) and severe periodontitis (Laine et al. 2005). However, there are also studies that have found no association (Heesen et al. 2001). Eng et al. (2004) reported that TNF-α production in a whole blood cell culture stimulated with *C. pneumoniae* or *C. trachomatis* was higher in subjects with the TT genotype than in those with the CC genotype. They also found that the prevalence of *C. pneumoniae* seropositivity was higher among the T allele carriers than among the carriers of the CC genotype (Eng et al. 2003). In addition, the T allele has been reported to be a risk factor of chronic *C. pneumoniae* infection of peripheral blood mononuclear cells in coronary artery disease patients (Rupp et al. 2004).

### 2.3.3 TLR-4

An $A_{896} \rightarrow G$ (Asp$^{299} \rightarrow$Gly) (rs4986790) polymorphism in the *TLR-4* gene has been suggested to cause LPS hyporesponsiveness of the TLR-4 receptor (Arbour et al. 2000, Schwartz 2001). Furthermore, carriage of the hyporesponsive G allele has been associated with e.g. a lower level of inflammatory molecules, an increased risk of severe infections, a reduced risk of atherosclerosis and coronary stenosis, increased carotid artery compliance (Kiechl et al. 2002, Hernesniemi et al. 2006, Hernesniemi et al. 2008), chronic periodontitis (Schröder et al. 2005), and an increased risk of Gram-negative septic shock (Lorenz et al. 2002b). Association of the G allele with asthma was reported in women who also carried the T allele of the *IL-4* $C_{-590} \rightarrow T$ polymorphism (Ådjers et al. 2005). The polymorphism has also been reported to possibly interfere with treatment response to a single disease-modifying antirheumatic drug in early rheumatoid arthritis (Kuuliala et al. 2006).

### 2.3.4 TLR-2

There is a $G_{2408} \rightarrow A$ (Arg$^{753} \rightarrow$Gln) (rs5743708) polymorphism in the *TLR-2* gene, the A (Gln) allele of which has been suggested to cause hyporesponsiveness to bacterial peptides and possibly increased risk of staphylococcal septic shock (Lorenz et al. 2000). Later on, several disease associations have been reported for this polymorphism, also. Carriage of the A allele has been associated with impaired immune response to *Borrelia burgdorferi* and protection from late stage Lyme disease (Schröder et al. 2005a), increased susceptibility to acute rheumatic
fever (Berdeli et al. 2005), recurrent febrile infections in children (Kutukculer et al. 2007), tuberculosis (Ogus et al. 2004), and coronary restenosis (Hamann et al. 2005).

2.3.5 IL-6

Numerous studies have focused on a G→C (rs1800795) promoter polymorphism in the IL-6 gene. In vitro studies have reported an association between the G allele and higher expression of IL-6 in HeLa cells transfected with G or C allele constructs (Fishman et al. 1998). It has also been reported that haplotypes (IL-6 polymorphisms G→A, G→C, G→C) containing the -174 C allele were associated with lower IL-6 production in LPS-stimulated whole blood, and that the -174 G allele containing oligonucleotide had a higher affinity for DNA-binding proteins than did the C allele containing oligonucleotide (Rivera-Chavez et al. 2003). The G allele and/or GG genotype have also been associated with higher serum levels of IL-6 e.g. in healthy subjects (Fishman et al. 1998) and in patients with primary Sjögren’s syndrome, but not in controls (Hulkkonen et al. 2001). On the other hand, higher expression of IL-6 has also been reported to associate with the CC genotype. The LPS-stimulated mononuclear cells of neonates with CC genotype were reported to secrete higher amounts of IL-6 than those with genotype CG or GG (Kilpinen et al. 2001). The CC genotype was associated with a lower serum IL-6 concentration in patients with small AAAs (Jones et al. 2001), and in neonates, but not in adults (Kilpinen et al. 2001). In addition, the GG genotype or G allele were associated with lower serum CRP levels in patients with small AAAs (Jones et al. 2001), and in neonates, but not in adults (Kilpinen et al. 2001). In obese men participating in a weight loss program, baseline serum CRP levels did not differ between the IL-6 genotypes, but after weight loss the CC genotype was associated with the highest serum levels and the smallest change in serum CRP levels (Eklund et al. 2006). Some studies have also suggested a different peak response of the IL-6 genotypes to stimulus (Brull et al. 2001), and some have found no association between the genotypes and IL-6 expression (Hulkkonen et al. 2000, Veres et al. 2002, Eklund et al. 2006, Sie et al. 2006, Hegedus et al. 2007).

The disease associations reported for the polymorphism include e.g. systemic-onset juvenile chronic arthritis (C allele as protective) (Fishman et al. 1998), Alzheimer’s disease (G allele as a risk) (Shibata et al. 2002), peripheral artery occlusive disease (GG genotype as a risk) (Flex et al. 2002), higher blood
pressure, and risk of coronary heart disease (C allele as a risk) (Humphries et al. 2001). An association between the polymorphism and longevity has also been addressed, and no difference was found in Finnish nonagerians when they were compared with blood donors (Wang et al. 2001), but in a follow-up study of the nonagerians the GG genotype and G allele frequencies were higher among the survivors than among the non-survivors (Hurme et al. 2005). Recently, in a relatively large (n = 2228) population-based study, the G allele was shown to be associated with higher serum levels of HDL and apoA1, higher carotid artery compliance, and lower systolic and diastolic blood pressure in men, suggesting that the G allele is associated with a beneficial profile of markers of early atherosclerosis (Hulkkonen et al. 2008). The polymorphism has also been associated with serum IgG autoantibody levels; carriers of the C allele have been reported to have remarkably lower serum levels of hHsp60 and Mycobacterium bovis Hsp65 IgG antibodies than the GG homozygotes (Veres et al. 2002, Pandey et al. 2004, KiszéI et al. 2006, Hegedus et al. 2007).

The discrepancy in these results may be at least partly due to different expression of IL-6 in different cell types and to the presence of other functional regulatory polymorphisms in the IL-6 promoter area (Terry et al. 2000). In addition, serum levels of IL-6 are affected by many factors that were differently addressed in the different studies. These factors include e.g. infections, body fat, smoking, time of day when a blood sample was taken, and stress (Gudewill et al. 1992, Steptoe et al. 2007, Sonnenberg et al. 2008). In addition, analysis of the association between IL-6 gene polymorphisms and serum CRP levels may be complicated by CRP polymorphisms like A−717→G (rs 2794521), C−26→T→A (rs3091244), G1109→C (rs1800947), C1444→T (rs1130864), and G1846→A (rs1205), which have been shown to regulate CRP expression (Hurme et al. 2007, Kivimäki et al. 2007, Eklund et al. 2008). Some of these polymorphisms have also been reported to associate with carotid artery compliance in men and with longevity in Finnish nonagenarians (Hurme et al. 2007, Eklund et al. 2008).

2.4 Obesity

Obesity in adults is defined by the body mass index (BMI), which is calculated as weight divided by height squared (kg/m²). The limits for normal weight are 18.5–24.9 kg/m², for overweight, 25.0–29.9 kg/m², and for obesity, ≥ 30.0 kg/m². According to the World Health Organization (WHO), the prevalence of obesity in the United States in 2005 was estimated to be 36.5% in men and 41.8% in women,
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and the prevalence of overweight was 75.6% in men and 72.6% in women. In Finland, the respective numbers in 2005 were 18.9% and 17.8% for obesity and 64.9% and 52.4% for overweight. (WHO 2008). Worldwide, 1.6 billion adults were overweight, at least 400 million were obese, and at least 20 million children under the age of five were overweight (WHO 2006). In Europe, the estimated increase in the rate of obesity over the last 10 to 15 years is approximately 30% (Berghöfer et al. 2008). Obesity is a risk factor of many diseases, including type II diabetes, cardiovascular disease, metabolic syndrome, degenerative joint disease, and some cancers (e.g. endometrial, breast, and colon cancer) (WHO 2006, Bessesen 2008). Causes of obesity include behavioral/environmental factors like diet, amount of exercise, socioeconomic status, and social networks (Christakis & Fowler 2007, Bessesen 2008). Lately, some genetic factors have also been described (Frayling et al. 2007, Saunders et al. 2007, Bouwman et al. 2008).

2.4.1 Inflammation in obesity

Obesity is associated with low-grade inflammation. In an obese state, increased infiltration and activation of macrophages into adipose tissue is seen. Adipocyte hypertrophy, local tissue hypoxia, and the presence of necrotic adipocytes in adipose tissue have been suggested as possible causes for this phenomenon (Cancello & Clément 2006). Adipose tissue also produces and secretes inflammatory molecules like TNF-α and IL-6, which are mainly produced by the macrophages of the stromal vascular fraction, but also by adipocytes (Hotamisligil et al. 1993, Cancello & Clément 2006, Desruisseaux et al. 2007, Sonnenberg et al. 2008). Adipose tissue also secretes adipokines, e.g. leptin, adiponectin, and resistin, which contribute to the regulation of energy homeostasis but can also play a role in processes like inflammation (Desruisseaux et al. 2007). Elevated serum levels of inflammatory markers such as IL-6 and CRP are associated with higher a BMI and obesity and can be reduced to some degree by weight loss (Yudkin et al. 1999, Festa et al. 2000, Lehtimäki et al. 2005, Eklund et al. 2006, Cartier et al. 2008). It has also been reported that serum leptin levels are independently associated with serum CRP levels, and that leptin may, either through IL-6 or directly, regulate CRP production (Viikari et al. 2007). Increased general levels of inflammatory markers again may contribute to development of obesity and other inflammation-related conditions like decreased insulin sensitivity, type 2 diabetes, metabolic syndrome, and CVD, which are all

2.4.2 Infections and obesity

One possible reason for chronic low-grade inflammation seen in obesity is the presence of microbial agents/products that promote inflammatory reactions. Several studies have reported an association between microbial infections or markers of infection and an elevated BMI or obesity. Despite these associations, it is not clear if obese people are more susceptible to infections or if infection predisposes to weight gain. Mice with diet-induced obesity have been shown to have impaired immune response to Porphyromonas gingivalis infection and less efficient clearance of the pathogen when compared with lean mice (Amar et al. 2007). In addition, it has been suggested that obese individuals may have impaired lymphocyte response when compared with non-obese controls (Tanaka et al. 1993), and one study has reported that overweight children may have higher susceptibility to acute respiratory infections than children with a low BMI (Jedrychowski et al. 1998). On the other hand, several infectious agents have been reported to possibly promote obesity; these organisms include canine distemper virus (CDV), Rous-associated virus-7 (RAV-7), Borna disease virus (BDV), scrapie agent, avian adenovirus SMAM-1, and some human adenoviruses like Ad-36 (Dhurandhar 2001, Pasarica et al. 2006) and Ad-5 (So et al. 2005). In humans, an association between obesity and viral infection has been reported at least for SMAM-1 and Ad-36. SMAM-1 antibody-positive, obese persons had a higher body weight and BMI than antibody-negative obese persons (Dhurandhar et al. 1997). Ad-36 antibody-positive persons had a higher BMI than antibody-negative persons, especially among the obese group but also among the non-obese group (Atkinson et al. 2005). In addition, Ad-36 infects adipocytes and induces adipogenesis in primary human adipose-derived stem cells (Rogers et al. 2007).

A few studies have also addressed the association between seropositivity for C. pneumoniae and an elevated BMI. Ekesbo et al. (2000) reported that among hypertensive and normotensive subjects, the group with combined seropositivity for Helicobacter pylori and C. pneumoniae had a higher BMI than the seronegative group. However, this difference did not reach statistical significance in a final multiple regression analysis. In a study population of coronary heart disease patients and controls, Dart et al. (2002) reported a higher BMI in a C. pneumoniae IgG seropositive group when compared with a seronegative group.
In a study by Thjodleifsson et al. (2007), C. pneumoniae IgG-positive individuals had a higher prevalence of overweight and a higher BMI than did IgG-negative ones. In addition, Karppinen et al. (2003) found that among patients with sciatica, those with chronic C. pneumoniae infection (IgG ≥ 128 and/or IgA ≥ 32 in two serum samples taken at least one month apart) had a higher BMI than did those with no signs of infection (IgG ≤ 16 and IgA ≤ 8). Saikku et al. (2004) have also reported an association between an elevated BMI and IgG and IgA antibodies at the age of 32 years in a Northern Finland Birth Cohort. However, opposite results have also been reported; in a study by Kaftan and Kaftan (2000), no association between C. pneumoniae IgG positivity and BMI was found in a study population of patients with coronary artery disease and healthy controls. Koziolek et al. (2008) found no association between past C. pneumoniae infection (64 ≤ IgG ≤ 256) and BMI, either. However, higher C. pneumoniae seroprevalence was observed among obese women with body fat mass and % of body fat values above the median than among those with values below the median.

Two recent studies have also shown that C. pneumoniae is capable of infecting murine preadipocytes and adipocytes (Shi et al. 2008) and human adipocytes derived from subcutaneous pre-adipocytes (Bouwman et al. 2008). In addition, the pathogen was found in preadipocytes in the adipose tissue of C. pneumoniae-infected LDLR−/− mice (Shi et al. 2008).

Besides infectious agents, also normal microbial flora may play a role in obesity. The composition of the gut microbiota of obese (ob/ob) mice was shown to be different from that of lean mice, and the microbiota of the obese mice were more effective in harvesting energy from the diet than were those of the lean mice. In addition, a transfer of the microbiota from obese mice to germ-free mice caused a higher percentage increase in body fat of the recipients when compared with mice that received microbiota from lean mice. This difference was not due to chow consumption. (Turnbaugh et al. 2006.) The same study group also demonstrated that obese persons have a decreased relative proportion of Bacteroides to Firmicutes species in their gut when compared with lean persons, and that this proportion increases with weight loss (Ley et al. 2006). In a recent study with mice, a high-fat diet was shown to cause a low-rate, chronic increase in the plasma concentration of LPS (termed metabolic endotoxemia). This increase in LPS levels was associated with increased body and fat deposit weight, fasted glycemia, and inflammation. In this study, Gram-negative gut microbiota was considered to be the source of LPS, and a high-fat diet was shown to cause changes in the composition of gut microbiota. (Cani et al. 2007.) In their
subsequent study Cani et al. (2008) showed that a high-fat diet increased gut permeability, and that this effect was restored by antibiotic treatment. Antibiotics also decreased plasma levels of LPS and occurrence of inflammation. The authors concluded that gut microbiota may regulate intestinal permeability, which again affects the metabolic endotoxemia and occurrence of metabolic disorders.

2.5 Cardiovascular diseases

Cardiovascular diseases (CVD) include e.g. coronary heart disease, cerebrovascular disease, hypertension, peripheral artery disease, rheumatic heart disease, congenital heart disease, and heart failure. Globally, CVD is the most common cause of death, and it has been estimated that in 2005, 17.5 million people died of CVD. (WHO 2007.) The most important single contributor to CVD is atherosclerosis. The individual risk of atherosclerosis and CVD is formed and modified by many factors, including e.g. smoking, unhealthy diet, physical inactivity, elevated homocysteine concentration, high levels of LDL and the presence of modified LDL, hypertension, metabolic syndrome, obesity, diabetes, alcohol misuse, infectious agents, gender, age, and genetic predisposition (Libby 2002, Alho et al. 2004, Pöllänen et al. 2005, Henttonen et al. 2007, Cubbon et al. 2008, Humphries et al. 2008, Mozaffarian et al. 2008). Atherosclerosis is also associated with the presence of inflammatory markers like IL-6, CRP, fibrinogen and matrix metalloproteinases in serum (Renko et al. 2004, Ferri et al. 2006). Moderately elevated serum levels of CRP are thought to be an early sign of atherosclerosis and have proved to be a relatively robust marker of cardiovascular risk (Koenig et al. 1999, Ridker et al. 2002, Zimmerman et al. 2003).

2.5.1 Formation of atherosclerotic lesions

It is thought that the first step of the atherosclerotic process is endothelial dysfunction resulting from an injury of the vascular wall. An injury may develop as a result of e.g. the presence of modified LDL, hyperlipidemia, free radicals caused by smoking, hypertension, or infectious agents. In certain areas or arteries like branches, bifurcations, and curvatures, blood flow is altered, showing e.g. reduced shear stress and increased turbulence. These areas are more prone to lesion formation than are areas with orderly blood flow. At these sites, permeability of the endothelium to plasma constituents like lipoproteins increases, and endothelial cells also express different adhesion molecules like vascular cell-
adhesion molecule 1 (VCAM-1), E-selectin, P-selectin, and inter-cellular adhesion molecule 1 (ICAM-1), and chemokines like monocyte chemotactic protein 1 (MCP-1) and IL-8. As a result, the adhesiveness of monocytes and T-cells to the endothelium increases, and these cells migrate to the subendothelial layer. (Ross 1999, Libby 2002.)

In lesions, monocytes mature to macrophages expressing scavenger receptors that bind modified lipoproteins like oxidized LDL. Accumulation of lipid droplets in the cytoplasm causes macrophages to form foam cells. Early atherosclerotic lesions (fatty streaks) are characterized by accumulation of lipids, monocytes, and T cells to the arterial wall. In later phases, growth factors and cytokines secreted by macrophages stimulate migration and proliferation of smooth muscle cells to the area of inflammation. Together with proteoglycan and loosely scattered collagen fibrils, smooth muscle cells form a fibrous cap on a lesion. Activated macrophages also present antigens to T cells causing them to activate. T cells again secrete e.g. IFN-$\gamma$, which may inhibit collagen production by smooth muscle cells and further activate macrophages. Helper T cells can polarize to secreting either $T_{H1}$ or $T_{H2}$ cytokines. In general, however, $T_{H1}$ cells predominate in atheroma. If the inflammatory response fails to remove or neutralize the offending agents, then inflammation, accumulation of cells and lipids in the lesion, and expansion of the lesion may continue. In advanced lesions, under a fibrous cap, there is a layer of accumulated lipids, leukocytes, and debris that may form a necrotic core. (Ross 1999, Libby 2002.)

By producing matrix metalloproteinases (MMPs), macrophages also play a role in degradation of the extracellular matrix and thinning of the fibrous cap. A rupture of the plaque may occur in areas where the fibrous cap is thin. In addition, degradation of the extracellular matrix may lead to hemorrhaging from the plaque microvessels or from the lumen of the artery. These may result in thrombus formation, possibly leading to formation of an occlusive blood clot and ischemia. Depending on the location of the clotted artery, ischemia may lead to e.g. occlusive peripheral arterial disease, transient ischemic attack, ischemic stroke, angina, and myocardial infarction. (Ross 1999, Libby 2002, Donnan et al. 2008.)

2.5.2 Abdominal aortic aneurysm

In an abdominal aortic aneurysm (AAA), a dilatation rather than an occlusion of the aorta is seen. An AAA is characterized by inflammation and degenerative changes in elastin and collagen in the aortic wall. The normal diameter of the

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abdominal aorta is 15–24 mm, and a diameter higher than 30 mm is considered an aneurysm. Male gender, age, smoking, and family history of AAAs have been reported as risk factors of an AAA. In addition, a strong association between atherosclerotic diseases and AAAs has been suggested. Many AAAs are asymptomatic and they are often found during a clinical examination done for some other reason, or after rupture. Rupture of an AAA is associated with high mortality; approximately half of the patients die before admission to hospital and the mortality rate during surgery is ~50%. (Satta et al. 1995, Jousimaa 2004, Wanhainen et al. 2005.)

2.5.3 Infections in CVD

For many microbes, including C. pneumoniae, Helicobacter pylori, several dental pathogens like Porphyromonas gingivalis, Prevotella intermedia, and Aggregatibacter actinomycetemcomitans, cytomegalovirus, and herpes simplex virus type 1, an association with CVD has been reported (Kusters & Kuipers 1999, Leinonen & Saikku 2002, Meurman et al. 2004, Grahame-Clarke 2005, Watson & Alp 2008). Seropositivity for these pathogens has been associated with CVD and many of them have also been detected directly in atherosclerotic plaques. Studies that have used broad range bacterial PCR have reported that bacterial DNA is commonly found in samples of diseased vascular tissue. Detection of DNA of several different species, many of which have not been previously studied in the context of CVD, has been reported. However, C. pneumoniae DNA is not usually detected with the broad range PCRs, possibly since the primers used in them may not efficiently amplify C. pneumoniae DNA. (Lehtiniemi et al. 2005, Marques da Silva et al. 2006, Ott et al. 2006, Renko et al. 2008.) Microbes may end up in the circulation from an oral cavity or other mucosal surfaces like the gut and promote atherosclerosis e.g. by molecular mimicry and by inducing both general and local inflammation. It has also been proposed that the combined effect of different infections, i.e. pathogen burden, plays a role on the pathogenesis of atherosclerosis. (Ludewig et al. 2004.)
2.5.4 C. pneumoniae and CVD

Seroepidemiological studies

The first report of an association between elevated levels of serological *C. pneumoniae* infection markers and CVD was based on observations in Finland. Elevated levels of *C. pneumoniae* IgG and/or IgA antibodies were present in the serum of 40–60% of the patients with acute myocardial infarction or chronic coronary heart disease, whereas they were seen in the serum of 10–15% of controls. (Saikku *et al.* 1988.) Chlamydial LPS-containing circulating immune complexes were also reported to be more common among patients with acute myocardial infarction (57%) than among the random controls (12%) or patient controls (10%) (Leinonen *et al.* 1990). In the Helsinki Heart Study, the presence of elevated IgA antibodies (titre ≥ 64) together with cLPS immune complexes in serum 3–6 months prior to infarction was shown to be associated with a 2.6-fold higher risk of myocardial infarction (Saikku *et al.* 1992). Since then, several other studies have also found an association between *C. pneumoniae* seropositivity and CVDs, like coronary artery disease, myocardial infarction, stroke, stenosis, transient ischemic attack, and angina pectoris (Watson & Alp 2008). In prospective meta-analysis studies of coronary heart disease, an odds ratio (95% CI) of 1.15 (0.97–1.36, NS) was reported for *C. pneumoniae* IgG positivity (Danesh *et al.* 2000), and 1.25 (1.03–1.53) for IgA positivity (Danesh *et al.* 2002). In a meta-analysis of atherosclerosis (coronary artery disease, stroke, or peripheral arterial disease) a slightly higher association was found; in the whole study the OR (95% CI) for IgG positivity was 1.6 (1.3–2.0), but in prospective studies it was 1.1 (0.8–1.4, NS) and in cross-sectional studies it was 2.0 (1.5–2.6) (Bloemenkamp *et al.* 2003). In many of the studies included in the meta-analyses the populations studied were relatively old, and thus prevalence of *C. pneumoniae* seropositivity in them was high and not necessarily due to persistent infection. In addition, the quality of serology in some of the publications used for meta-analyses may not have been first class. Indeed, it has been reported that in otherwise healthy 11-year-old children, persistent IgG and/or IgA seropositivity is associated with increased abdominal aorta intima-media thickness, a first sign of atherosclerosis, when compared with seronegative or only transiently seropositive children (Volanen *et al.* 2006).
Detection of *C. pneumoniae* in vascular tissue

The presence of *C. pneumoniae* antigens and/or DNA in lesions of infected animals has been reported by several studies with rabbits and mice (Fong et al. 1997, Laitinen et al. 1997, Fong et al. 1999, Campbell et al. 2000, Erkkilä et al. 2004, Ezzahiri et al. 2006). A study with dogs also demonstrated the presence of both *C. pneumoniae* and *C. psittaci* antigens in atheromatous lesions of aortas, coronary arteries and splenic arteries of dogs over 10 years of age with systematic atherosclerosis, but not in younger control dogs. The study also reported finding chlamydia-like structures in vascular endothelial and smooth muscle cells by electron and immunoelectron microscopy, and *C. pneumoniae* DNA in the spleen, heart, and kidney of a dog with atherosclerosis. (Sako et al. 2002.)

*C. pneumoniae* has also been demonstrated in atherosclerotic tissue of humans by several different methods. Some studies have reported direct isolation of the pathogen by culture from the coronary artery (Ramirez 1996, Maass et al. 1998a) and the carotid artery (Jackson et al. 1997, Cuffini et al. 2006). Several studies have also reported detection of *C. pneumoniae* in vascular tissue by immunohistochemistry/immunocytochemistry (Kuo et al. 1995a, Jackson et al. 1997, Juvonen et al. 1997, Cochrane et al. 2005), PCR (Kuo et al. 1995a, Jackson et al. 1997, Juvonen et al. 1997, Maass et al. 1998a, Maass et al. 1998b, Cochrane et al. 2003, Cochrane et al. 2005, Cuffini et al. 2006), electron microscopy (Ramirez 1996, Juvonen et al. 1997, Shor & Phillips 2000, Cuffini et al. 2006, Borel et al. 2008), immunoelectron microscopy (Borel et al. 2008), and in situ hybridization (Ramirez 1996, Alakärppä et al. 1999). Detection of *C. pneumoniae* in healthy vascular tissue is rare, and most studies have reported no detection in their control samples of healthy tissue (Kuo et al. 1995a, Juvonen et al. 1997, Maass et al. 1998b).

Animal models

Several studies with rabbits and mice have reported that *C. pneumoniae* infection promotes atherosclerotic processes. In some studies, *C. pneumoniae* infection alone has been reported to be able to cause early atherosclerotic changes. In their study with New Zealand White rabbits fed on standard chow, Fong et al. (1997) reported that a single infection with *C. pneumoniae* (ATCC strain VR-1310) alone was enough to cause a fatty streak (histologic grade I) in the aorta of one animal and a histologic grade III lesion in the aorta of another rabbit. Both of these aortas
stained positive with *C. pneumoniae* immunohistochemistry and the pathogen was cultured from the aorta with a grade III lesion. In another, larger study Fong *et al.* (1999) also concluded that *C. pneumoniae* (strains VR-1310 and AR-39) infection alone can induce early atherosclerotic changes that resemble changes induced by a very low cholesterol diet in the aortas of rabbits. In a similar study with *C. pneumoniae* (strain Kajaani 7), animals with two repeated infections were shown to have intimal thickening or fibroid plaques resembling atherosclerosis in their aortas and in one animal calcified lesions were seen. However no atherosclerotic changes were observed in animals infected only once. (Laitinen *et al.* 1997).

Many studies, however, report that at least some cholesterol supplement in chow or hyperlipidemia induced either by diet or genetic background is needed in addition to *C. pneumoniae* infection in order to see any lesion development. In the study by Muhlestein *et al.* (1998), it was reported that three repeated infections with *C. pneumoniae* (strain VR-1310) accelerated intimal thickening in New Zealand White rabbits fed with cholesterol-supplemented chow and, interestingly, thickening could be prevented by weekly treatment with azithromycin. However, in this study there was no comparison between normal and cholesterol-supplemented chow. In rabbits, repeated intravascular injections of viable or heat-killed *C. pneumoniae* (strain AR-39) or *E. coli* LPS resulted in lesion formation in animals fed a high-cholesterol diet but not in animals fed a normal diet (Engelmann *et al.* 2006). In a study with LDLR-deficient B6,129 mice, repeated *C. pneumoniae* (strain AR-39) infection was shown to significantly enhance development of atherosclerotic lesions, but only when the mice were fed a 2% cholesterol-supplemented diet but not in animals fed a normal diet (Hu *et al.* 1999). In apoE-deficient mice fed a normal diet, repeated *C. pneumoniae* (strain AR-39) inoculations were shown to cause a significant increase in lesion area, but in C57BL/6J mice only inflammatory changes in the heart and aorta were seen (Campbell *et al.* 2000). It has also been reported by two studies that *C. pneumoniae* (strain Kajaani 7) infection together with a slightly (0.2%) cholesterol-supplemented diet contributes to the development of lipid lesions in the aortas of normocholesterolemic C57BL/6J mice (Erkkilä *et al.* 2004, Törmäkangas *et al.* 2005). In C57BL/6J mice fed a high-fat/high-cholesterol diet, repeated intraperitoneal injections with *C. pneumoniae* (strain VR-1355) resulted in a marked enlargement of atherosclerotic lesions in the aortic sinus (Ezzahiri *et al.* 2006).
However, not all animal studies have shown an association between *C. pneumoniae* infection and atherosclerosis. In ApoE-deficient mice (FVB and C57BL/6J backgrounds), no difference in the size of aortic root lesions between mice repeatedly infected with *C. pneumoniae* (strain Kajaani 7) and control mice was seen in animals fed either chow or a high-fat diet (Aalto-Setälä *et al.* 2001).

**Antibiotic treatment trials**

Several relatively small antibiotic treatment studies have reported a better outcome in patients with CVD if treated against *C. pneumoniae* infection. One study reported that in men that had survived a myocardial infarction, treatment with azithromycin (500 mg/day for three days or two such courses three months apart) decreased the risk of further cardiovascular events by fivefold when compared with a no-treatment/placebo group. In addition, treatment was reported to significantly lower serum *C. pneumoniae* IgG levels. (Gupta *et al.* 1997.) Roxithromycin treatment, again, has been reported to associate with a lower rate of triple and double secondary end points in patients with angina (Gurfinkel *et al.* 1999), and with less frequent detection of *C. pneumoniae* DNA in carotid plaques of carotid endarterectomy patients (Melissano *et al.* 1999). In addition, roxithromycin treatment was reported to prevent progression of peripheral arterial occlusive disease in *C. pneumoniae* seropositive men (Wiesli *et al.* 2002). The dosage and duration of antibiotic treatment in the roxithromycin studies were 150 mg twice a day for a mean of 24 days (Gurfinkel *et al.* 1999), 150 mg twice a day for a mean of 26 days (Melissano *et al.* 1999), and 300 mg a day for 28 days (Wiesli *et al.* 2002).

However, some smaller studies and recent large studies have failed to find any association between antibiotic treatment and the disease outcome. Azithromycin treatment was not found to have an effect on the number of clinical cardiovascular events in *C. pneumoniae* seropositive patients with CAD (Anderson *et al.* 1999), the number of primary end points in *C. pneumoniae* seropositive patients with previous myocardial infarction (O’Connor *et al.* 2003), or the number of primary end points in patients with documented stable coronary artery disease (Grayston *et al.* 2005). In these studies the dosage and duration of treatment were 500 mg a day for three days and then 500 mg a week for three months (Anderson *et al.* 1999), 600 mg a day for three days then 600 mg a week until week 12 (O’Connor *et al.* 2003), and 600 mg a week for one year (Grayston *et al.* 2005). Similarly, treatment with gatifloxasin (400 mg a day for the first two
weeks and an additional 10-day course a month for a mean of two years) had no significant effect on the rates of primary end points in patients hospitalized for acute coronary syndrome (Cannon et al. 2005).

Interestingly, many of the studies that looked for markers of C. pneumoniae infection as a response to antibiotic treatment found no changes (Anderson et al. 1999, Gurfinkel et al. 1999, O’Connor et al. 2003). Similarly, no changes were seen in serological markers of C. pneumoniae infection during/after doxycycline (100 mg a day for four months) treatment in men with coronary heart disease (Sinisalo et al. 1998), after azithromycin treatment (500 mg on the two first days then 250 mg a day until day 28) in patients that had undergone percutaneous coronary revascularization (Jackson et al. 1999), or after clarithromycin treatment (500 mg/day for a mean of 27 days before surgery) in patients with coronary artery disease before and after surgery (Berg et al. 2005). In addition, clarithromycin treatment did not affect the presence of MOMP or LPS antigens in the vascular specimens, and no C. pneumoniae DNA could be detected in any of the vascular specimens by two different PCR methods (real-time and industry-derived RUO-PCR) (Berg et al. 2005). These results may implicate that the antibiotics used did not eradicate persistent C. pneumoniae infection. On the other hand, C. pneumoniae may play an active role in early phases of the atherosclerotic process but not later when CVD has already been established. It has also been suggested that in tissue with advanced atherosclerosis, chlamydial components rather than whole bacteria are present (Meijer et al. 2000). In both cases antibiotic treatment of advanced atherosclerosis would not be expected to be beneficial. It may be that in order to be effective, treatment should be given after/during primary infections, as in rabbit models where azithromycin and gatifloxacin were found to be efficient in preventing induction of atherosclerosis by C. pneumoniae (Muhlestein et al. 1998, Jones et al. 2003). However, a study with 13-year-old children showed that use of antichlamydial antibiotics was associated with increased abdominal aorta intima-media thickness, and that thickening was further enhanced by the presence of an elevated CRP concentration (Volanen et al. 2008). This result implies that antibiotic therapy may not be beneficial in primary prevention of atherosclerotic cardiovascular diseases.
Mechanisms of pathogenesis

*Chlamydia pneumoniae* is capable of infecting and surviving in several different cell types important in the pathogenesis of CVD, including monocytes/macrophages, vascular endothelial cells, and smooth muscle cells (Gaydos 2000). The possible route from the lungs to vascular tissue is through bronchoalveolar macrophages infected during pulmonary *C. pneumoniae* infection. Moazed et al. demonstrated that in intranasally or intraperitoneally infected mice, *C. pneumoniae* was detectable by isolation, fluorescent staining, and/or PCR in either alveolar or peritoneal macrophages (respectively) and in peripheral blood mononuclear cells (PBMC). Furthermore, transmission of macrophages from infected mice to uninfected mice resulted in detection of *C. pneumoniae* DNA in the lungs, thymus, spleen, and abdominal lymph nodes of the recipient mice. (Moazed et al. 1998.) *C. pneumoniae* has also been detected in peripheral blood monocytes in humans, both in otherwise healthy blood donors (Boman et al. 1998, Bodetti & Timms 2000, Cirino et al. 2006) and in patients with CVD (Boman et al. 1998, Cochrane et al. 2005). *C. pneumoniae* is viable in infected monocytes but does not produce infectious progeny (Airenne et al. 1999), whereas in macrophages it has been shown to be capable of infective growth, though some inhibition was observed (Gaydos et al. 1996). Furthermore, it has been shown that *C. pneumoniae* can transfer from infected monocytes to endothelial cells (Lin et al. 2000) and smooth muscle cells (Puolakkainen et al. 2003), and *C. pneumoniae* growth in endothelial and smooth muscle cells is enhanced by co-cultured monocytes (Lin et al. 2000, Puolakkainen et al. 2003).

*C. pneumoniae* is also capable of inducing cellular reactions that are associated with pathogenesis of atherosclerosis. *C. pneumoniae* infection has been shown to increase adherence of monocytes/macrophages to endothelial cells (Molestina et al. 1999, Kalayoglu et al. 2001, May et al. 2003, Azenabor et al. 2005, Takaoka et al. 2008). Possible mechanisms of adherence include increased expression and/or activation of integrins like very late antigen-4 (VLA-4), leukocyte function-associated antigen-1 (LFA-1), and macrophage antigen-1 (Mac-1) (Kalayoglu et al. 2001, May et al. 2003, Takaoka et al. 2008). On the other hand, *C. pneumoniae*-infected macrophages have been reported to show altered composition of the plasma membrane, which increases their fluidity and may thus promote fragility and attachment to endothelial cells (Azenabor et al. 2005). In addition, *C. pneumoniae* infection in monocytes/macrophages induces production of inflammatory cytokines like TNF-α, IL-1β, and IL-6, increases
expression of LPS-receptor CD14 (Heinemann et al. 1996, Kaukoranta-Tolvanen et al. 1996b), and together with LDL induces macrophage foam cell formation (Kalayoglu & Byrne 1998b). *C. pneumoniae* can also induce smooth muscle cell proliferation either directly (Miller et al. 2000, Hirono et al. 2003, Rupp et al. 2005) or through soluble factors produced by infected endothelial cells (Coombes & Mahony 1999). In endothelial and vascular smooth muscle cells, infection increases activation of NF-κB and expression of e.g. chemokines like MCP-1 and IL-8, adhesion molecules like E-selectin, ICAM-1, and VCAM-1, inflammatory cytokines like IL-6, and coagulation factors like tissue factor (TF) (Kaukoranta-Tolvanen et al. 1996a, Dechend et al. 1999, Krüll et al. 1999, Molestina et al. 1999).

Many studies have reported that cLPS and Hsp60, in particular, play a role in the pathogenesis of cardiovascular diseases. Both of them have been reported to possibly increase adhesion of monocytes to endothelial cells (Kalayoglu et al. 2001, May et al. 2003), and have been shown to induce cell activation by stimulating the NF-κB pathway and production of inflammatory cytokines like TNF-α and IL-6. In addition, cHsp60 can induce production of matrix metalloproteinases (MMPs) by macrophages. (Ingalls et al. 1995, Kol et al. 1998, Kol et al. 1999, Heine et al. 2003.) It has also been shown that cHsp60 can induce LDL oxidation (LaVerda et al. 1999) and that cLPS can induce macrophage foam cell formation (Kalayoglu & Byrne 1998a). In addition to inflammation, cHsp60 may promote atherosclerosis by inducing cross-reactive immune responses against human Hsp60. Due to the high degree of conservation of Hsps between different organisms, microbial infections may promote production of anti-Hsp antibodies that protect the host from infection but are also cross-reactive with the host Hsps (Wu & Tanguay 2006). Interestingly, cHsp60 has been reported to colocalize with human Hsp60 in macrophages of human atheromas (Kol et al. 1998), and *C. pneumoniae* infection in mice has been shown to increase the serum levels of mouse Hsp60 IgG antibodies (Erkkilä et al. 2004, Törmäkangas et al. 2005). In addition, simultaneous presence of human Hsp60 IgA antibodies, *C. pneumoniae* IgA antibodies, and elevated CRP is associated with an elevated risk of coronary events (Huittinen et al. 2002). On the other hand, serum cHsp60, but not hHsp60 IgG antibodies, were reported to associate with coronary artery disease (Mahdi et al. 2002).

In addition to promoting inflammatory reactions, *C. pneumoniae* infection may promote pathogenesis of CVD by modifying the serum lipid profile towards more atherogenic; *C. pneumoniae* seropositive persons have been demonstrated to
have higher serum triglyceride and total cholesterol concentrations and lower HDL concentrations and lower HDL-to-total-cholesterol ratios than seronegative persons (Laurila et al. 1997, Murray et al. 1999). Fig. 3 shows a summary of the different mechanisms through which C. pneumoniae may promote atherosclerosis and CVD.

![Diagram showing different mechanisms through which chlamydial Hsp60 and LPS may promote pathogenesis of atherosclerosis. Modified after Leinonen & Saikku (2002), Watson & Alp (2008).]

Fig. 3. Different mechanisms through which chlamydial Hsp60 and LPS may promote pathogenesis of atherosclerosis. Modified after Leinonen & Saikku (2002), Watson & Alp (2008).
3 Aims of the study

The overall aim of this work was to study the role of persistent *C. pneumoniae* infection, inflammatory and innate immunity markers, and selected gene polymorphisms in cardiovascular diseases. In addition, our aim was to develop new methods both for genotyping the gene polymorphisms of interest and for detecting *C. pneumoniae* infection. The specific aims of this work were:

1. To develop a faster and easier method for genotyping the *LBP* C\(_{1341}\)→T (Leu\(_{436}\)→Phe) polymorphism.
2. To study how serum markers of inflammation and innate immunity are correlated in a normal population and whether serum levels are regulated by the selected gene polymorphisms.
3. To study how markers of chlamydial infection are associated with serum markers of inflammation and innate immunity in a normal population.
4. To study if the selected gene polymorphisms are associated with markers of chlamydial infection.
5. To set up a method for detecting cLPS from atherosclerotic tissue.
6. To compare the performance of the cLPS method in detecting chlamydial infection with several different PCR methods and ISH in atherosclerotic tissue samples.
7. To study if serum cLPS concentration is associated with BMI.
4 Materials and methods

4.1 Study subjects and specimens

Our study populations consisted of Finnish blood donors from the city of Oulu and patients with CVD recruited at the Helsinki University Central Hospital during the years 2002–2004.

There were 393 individuals in the blood donor group, including 271 men and 122 women. From this population, buffy coats and sera were collected during the years 2001 and 2002 and obtained from the Finnish Red Cross Blood Service (Permission number 48). Sera were available from all but one and DNA from all the blood donors. The cells were stored at −80 °C until DNA extraction and the DNA and sera at −20 °C, until analysis.

The CVD study group consisted of 110 patients with CAR, 50 patients with AAA, and 22 patients with AOD. In this study population, carotid surgery was performed on symptomatic patients with a moderate (50–69%) or high-grade (70–99%) common and/or internal carotid artery stenosis. Carotid endarterectomy was performed under general anesthesia in a routine manner, with transcranial doppler monitoring, selective shunting and selective patching. Surgery for an AAA or AOD was performed through laparotomy. Aneurysm operations were all elective repairs for AAAs with a mean maximum diameter of 61.6 mm (40–112 mm). Three aneurysm patients had disabling claudication, as well. All the patients with aorto-occlusive disease had disabling claudication caused by aortoiliac lesions so extended that endovascular treatment was not feasible. All the patients gave their informed consent. The local Ethics Committee at Helsinki University Central Hospital approved the study protocol.

All the blood samples were collected from an upper arm vein before the induction of anesthesia in the operation theatre and prepared within 30 min from arterial clamping. Sera were then separated, divided to aliquots and stored at −20 °C until analysis. Tissue samples were collected during the surgery; calcified areas of the plaque were excluded, if possible. The tissue samples for DNA extraction were stored at −70 °C: samples were also collected for paraffin-embedded sections, TEM, and IEM and handled and stored as described later in the context of each method.
4.2 Measurements of serum cholesterol and triglycerides, and markers of inflammation and innate immunity (studies II and IV)

The serum levels of LBP (Hycult HK315, Hycult Biotechnology, Uden, The Netherlands), sCD14 (Hycult HK320, Hycult Biotechnology), IL-6 (PeliKine compact® human IL-6 elisa kit, Sanquin Reagents, Amsterdam, Netherlands), and hsCRP (hs-CRP (IEMA), MedixBiochearma, Helsinki, Finland) were measured with commercial EIA kits (II and IV). The measurements were done according to the manufacturers’ instructions, except that the samples were not done as duplicates. The cholesterol and triglyceride values were measured from the arterial blood samples in a Helsinki University Central Hospital laboratory using routine photometric, enzymatic analysis for total cholesterol, HDL, and triglycerides (TG) and a Friedewald formula for LDL (IV).

4.3 DNA extraction (I, II, III)

DNA was extracted from the buffy coats of the blood donors using the tissue protocol of the QIAamp DNA Mini Kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions; the amount of starting material was variable (I, II). After extraction, the DNA concentration was determined photometrically.

For study III, several different DNA extraction methods were used. A single 50-mg piece and/or five 5-mg pieces were cut from each carotid artery tissue sample. Separate disposable scalpels and Petri dishes were used for the different samples. The five 5-mg pieces were taken from different parts of the tissue and treated as separate samples in DNA extraction. To homogenize the tissue samples, 400 μL of homogenization buffer (0.05 mol/l Tris, 0.05 mol/l NaCl, 0.05 mol/L EDTA, 1% sodium dodecyl sulfate, pH 8.0) and 20 μL of 10 mg/ml proteinase K (Sigma, St. Louis, MO) were added to the samples. Homogenization was done at a speed of 6 m/s for 5 × 30 sec using BIO101® System’s lysing matrix D tubes (Qbiogene, Carlsbad, CA) and FastPrep homogenizer FP120 (Thermo Electron Oy, Vantaa, Finland). The samples were allowed to cool on ice for 5 min between the homogenization steps. A 100-μl amount of 10 mg/ml proteinase K (Sigma) was then added, and the samples were incubated at +56 °C overnight. After centrifugation at 18 330 × g for 10 min, 400 μL of the lysate was used for DNA extraction with the QIAamp® DNA mini kit (QIAGEN) tissue protocol according to the manufacturer’s instructions. The elution volume was either 200 or 100 μL, depending on the amount of starting material (50 or 5 mg of tissue, respectively). At least every 16th sample in DNA extraction was a
negative control that was also tested in a PCR. Unless mentioned otherwise in the PCR protocol, the DNA samples and negative controls were kept at −20 °C until PCR analysis.

Pieces of plaque were fixed in 10% formalin and fused in paraffin. From these tissue samples, 5-μm-thick sections were cut and two or four sections per sample were used as a starting material for DNA extraction (III). Paraffin was removed from the sections as instructed in the QIAamp® DNA mini kit (QIAGEN) protocol A for isolation of genomic DNA from paraffin-embedded tissue. Because the amount of starting material was small, only 600 μl of both xylene and ethanol was used. The resulting pellet was suspended in 15 μl of buffer ATL. For DNA extraction, the QIAamp DNA micro kit (QIAGEN) protocol for the isolation of genomic DNA from laser-microdissected tissues was used according to the manufacturer’s instructions. Negative controls and storage of the DNA were as described above.

4.4 Genotyping (I, II)

A real-time PCR method for genotyping the $LBP_{C_{1341}→T}$ (Leu$^{436}→$Phe, rs2232618) polymorphism was developed (I). The primers and probes were designed by and obtained from Tib MolBiol GmbH (Berlin, Germany). The PCR was carried out using a LightCycler real-time PCR machine 1.2 (Roche Diagnostics GmbH, Mannheim, Germany) and software version 3.5. The reaction volume was 20 μL with 2.875 mM of MgCl2, 0.5 μM of both primers, 0.2 μM of both probes and 2 μL of 10×LightCycler - FastStart DNA Master Hybridization Probes buffer (Roche Diagnostics GmbH), and about 40 ng of template DNA. The PCR protocol consisted of initial denaturation at 95 °C for 10 min, followed by 60 cycles of denaturation (95 °C for 0 sec, 20 °C/s), annealing (52 °C for 5 sec, 20 °C/s) and elongation (72 °C for 10 sec, 5 °C/s). This was followed by a melting curve analysis consisting of 1 cycle at 95 °C for 0 sec (20 °C/s), 45 °C for 60 sec (20 °C/s), and a temperature rise to 90 °C at a slope of 0.1 °C/s with continuous measurement of fluorescence. To confirm the results of our LightCycler genotyping, nine samples representing different genotypes were sequenced in both directions (by Macrogen Inc. 10F World Meridian Center, 60-24 Kasan-dong, Kumchonkum, Seoul, Korea).

In study II, the polymorphisms studied were $LBP_{C_{1341}→T}$ (Leu$^{436}→$Phe, rs2232618), $CD14_{C_{-260}→T}$ (rs2569190), $TLR-4_{A_{896}→G}$ (Asp$^{299}→$Gly, rs4986790), $TLR2_{G_{2408}→A}$ (Arg$^{753}→$Gln, rs5743708), and $IL-6_{G_{-174}→C}$ (rs1800795). Genotyping of the $LBP$ polymorphism was performed in the context of study I. To genotype the other polymorphisms, real-time PCR methods were
set up according to previously published methods (Heesen et al. 2000, Bertsch et al. 2001, Heesen et al. 2001, Heesen et al. 2003, Hamann et al. 2004). Table 2 shows the names and oligonucleotide sequences of the primers and probes used in the genotyping PCRs. To clarify the naming of the oligonucleotides, all the primers were named either for or rev and all the probes either dete or anch. All the primers and probes were obtained from Tib MolBiol GmbH, except for those used in CD14 genotyping, which were obtained from Proligo (Boulder, CO, USA).

Table 2. Primers and probes used in genotyping PCR reactions.

<table>
<thead>
<tr>
<th>Study</th>
<th>Oligonucleotide name</th>
<th>Sequence (5’→3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, II</td>
<td>LBPfor</td>
<td>TTTGCTTTTCCCAAGCGTT</td>
<td>Study I</td>
</tr>
<tr>
<td></td>
<td>LBPrev</td>
<td>GAGCCTGTTTTCCAAGTCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LBPdete</td>
<td>CTATTACATCCTTAACCCCTAC-FL</td>
<td>Heesen et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>LBPanch</td>
<td>Red 640²-CCAAGTTCAATGTAAGAATCCTGTGGA-FL³</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td>II</td>
<td>CD14for</td>
<td>GGTGCCAACAGATGAGGTTCA</td>
<td>Heesen et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>CD14rev</td>
<td>CTTCGGCTGCTCAGTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD14dete</td>
<td>Red 640²-TTCCTGTTACGCCCCCT-FL³</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>CD14anch</td>
<td>GGAGACACAGAACCTAGTGCTGCA-FL</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td>II</td>
<td>TLR4sense</td>
<td>AAGAAATTAGGCTTTCAAGCT</td>
<td>Heesen et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>TLR4antis.</td>
<td>ACCCTTTCAATAGTCACACTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TLR4dete</td>
<td>ACTACCTCGATGATATTGGACTTATT-FL³</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>TLR4anch</td>
<td>Red640²-AATTGTTGACAAATGTTTCTCATTTC-FL³</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td>II</td>
<td>TLR2for</td>
<td>AGTGAGCAGGGATGCCTACT</td>
<td>Hamann et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>TLR2rev</td>
<td>GACTTTATCGACAGCTCTACATTAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TLR2dete</td>
<td>CAAGCTGCAAGAAGATGGAACACAAG-FL</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>TLR2anch</td>
<td>Red640²-CCTACCTGGATGCGCCCAGGAC-FL³</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td>II</td>
<td>IL-6for</td>
<td>TTACTCCTGGTCAAGACATGCCA</td>
<td>Bertsch et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>IL-6rev</td>
<td>ATGAGCCTCAGACATCTCCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-6dete</td>
<td>Red640²-GTGCTTGCGATGCTAAGA-P³</td>
<td>Heesen et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>IL-6anch</td>
<td>CTAGCTGCACTTTCCCTAGT-FL³</td>
<td>Heesen et al. (2001)</td>
</tr>
</tbody>
</table>

¹Fluorescin label, ²LC Red640 label, ³Phosphate

For CD14 genotyping, the previously published protocol (Heesen et al. 2000, Heesen et al. 2001) was modified as follows. FastStart DNA Master Hybridization probe buffer (Roche Diagnostics GmbH) was used, and the final MgCl2 concentration was 2.5 mM in a reaction volume of 20 μL. Initial denaturation at 95 °C was prolonged to 10 min and the denaturation phase during cycling, to 5 sec.
For TLR-4 genotyping, the original protocol (Heesen et al. 2003) was also slightly modified. FastStart DNA Master Hybridization probe buffer (Roche Diagnostics GmbH) was used, and the concentration of both primers was 0.5 μM in a 20 μL reaction volume. Initial denaturation at 95 °C was prolonged to 10 min and cycling was performed at 95 °C for 2 sec, at 49 °C for 15 sec, and at 72 °C for 20 sec. The TLR4 probes were not labeled as in the original publication, since there both the Red-640 and fluorescein labels were placed in the same probe (Red640-ACT ACC TCG ATG ATA TTA TTG ACT TAT T-fluorescein), probably due to a typing mistake.

The modifications for the TLR-2 genotyping (Hamann et al. 2004) included adding a phosphate to the 3’ end of the anchor probe to prevent priming by the probes and changing the primer concentration to 0.5 μM and the final MgCl2 concentration to 3.0 mM.

The genotyping PCR for the IL-6 polymorphism was as described in the original publication (Bertsch et al. 2001).

The genotyping PCRs were performed with the LightCycler real-time PCR machine 1.2 and 2.0 (Roche Diagnostics) and the software versions 3.5 and 4.0 (Roche Diagnostics). A melting curve analysis was performed in all the PCRs and the genotyping results were interpreted according to derivative melting curves [μd(F2 / F1) / dt] blotted against temperature. All the runs contained water samples as negative controls.

4.5 Detection of C. pneumoniae (II, III, IV)

4.5.1 PCR methods (III)

Three different PCR assays were used to detect C. pneumoniae DNA: a quantitative real-time PCR assay (16S LC-PCR) (Reischl et al. 2003), a nested PCR assay combining conventional PCR and real-time PCR (semiconventional Pst PCR) (Ciervo et al. 2003, Törmäkangas et al. 2005), and a conventional nested PCR assay with nonradioactive hybridization to detect the correct PCR product (conventional Pst PCR) (Campbell et al. 1992, Maass et al. 2000). Table 3 shows the names and oligonucleotide sequences of the primers and probes used in C. pneumoniae detection.

The 16S LC-PCR amplified a region of the 16S rRNA gene and was performed as described in the original publication (Reischl et al. 2003). The primers and
probes used in this PCR were CpnA and CpnB, and CP16FL and CP16Red640, respectively.

Table 3. Primers and probes used in the detection of C. pneumoniae DNA.

<table>
<thead>
<tr>
<th>Study</th>
<th>Oligonucleotide name</th>
<th>Sequence (5'→3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>HL-1</td>
<td>GTTGTTCATGAAGGCCTACT</td>
<td>Campbell et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>HR-1</td>
<td>TGATAACCTACGGTGTT</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>In-1</td>
<td>AGTTGAGCATATTCGTGAGG</td>
<td>Maass et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>In-2</td>
<td>TTTATCTCGTGTCGCCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HM-1</td>
<td>DIG\textsuperscript{1}-GTGTCATTCGCAAGGTTAA</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>CP1-Flu</td>
<td>CTCTTAGGGCAACGTAAGC-FL\textsuperscript{2}</td>
<td>Ciervo et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>CP1-LC</td>
<td>Red640\textsuperscript{1}-TAACCTGGCGAATGACACCA-P\textsuperscript{4}</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Pstfor</td>
<td>AAACGGCATTACACGGCTA</td>
<td>Törnäkangas et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Pstrev</td>
<td>GGGAACGATTTGGAACAA</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>CpnA</td>
<td>TGACAACGTGAAGAAATACGC</td>
<td>Gaydos et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>CpnB</td>
<td>CGCCCTCCTCCTATAAAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP16FL</td>
<td>GTAGCAAGATGGTGGAGATGGAGCAA-FL\textsuperscript{2}</td>
<td>Reischl et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>CP16Red640</td>
<td>Red640\textsuperscript{1}-TCCTAAAGCTAGCCCCAGTT-C-P\textsuperscript{4}</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1}Digoxigenin label, \textsuperscript{2}Fluorescein label, \textsuperscript{3}LC Red640 label, \textsuperscript{4}Phosphate

The semiconventional Pst PCR first amplified a 192-bp product of the C. pneumoniae Pst1 fragment with the outer primer pair pstfor and pstrev. To prevent contamination of this step with the PCR products of the first step, dUTP (2'-deoxyuridine 5'-triphosphate) (Roche Diagnostics GmbH) was used instead of dTTP (2'-deoxythymidine 5'-triphosphate), and uracil-DNA N-glycosylase (UNG) enzyme (Roche Diagnostics GmbH) was added to the reaction. The PCR program included initial incubation for 10 min at 20 °C for the UNG enzyme and 2 min at 95 °C for denaturation of the enzyme. The first-step PCR products were diluted 1:100, and 8 μl of these dilutions were used for the second step of the PCR done with the LightCycler real-time-PCR instrument (Roche Applied Science). In this phase, adapted from a previously published protocol (Ciervo et al. 2003), the inner primers ln-1 and ln-2 amplified a 128-bp product, and the product was recognized specifically by the probes CP1-Flu and CP1-LC. The reaction volume was 20 μl with 3.5 mM MgCl\textsubscript{2}, 0.4 μM of each primer, 0.2 μM of each probe, and 2 μl of 10 x FastStart DNA Master Hybridization Probes mixture. The PCR program consisted of preincubation at 95 °C for 10 min, 55 cycles at 95 °C for 10 sec, 57 °C for 8 sec, and 72 °C for 10 sec.
The conventional Pst PCR was slightly modified from a previously published method (Campbell et al. 1992, Maass et al. 2000). The PCR mix contained 200 μmol/L of each dNTP, 1 μmol/L of both primers HL-1 and HR-1 (TIB MOLBIOL, Berlin, Germany), 2.5 U of Taq polymerase (Promega Biosciences, San Luis Obispo, CA), 3.5 mmol/L MgCl₂ (Promega Biosciences), and 5 μL of 10× PCR buffer (Promega Biosciences). The nested step used the same PCR conditions, with the exception that the primers were In-1 and In-2 (TIB MOLBIOL), and 2.5 μL of the PCR product was used as a template. The final PCR products were run on 1.5% agarose gel, blotted on a positively charged nylon membrane (Roche Diagnostics, Penzberg, Germany), and cross-linked with ultraviolet light. For prehybridization and hybridization, DIG Easy Hyb solution (Roche Diagnostics) containing 0.1 mg/mL fish DNA (Roche Diagnostics, Penzberg, Germany) was used according to the manufacturer’s instructions. Prehybridization was performed at +42 °C for 60 min and hybridization at +42 °C overnight. The hybridization solution contained 0.63 pmol/mL of 5′ digoxigenin-labeled HM-1 probe (TIB MOLBIOL) that was detected using a DIG Nucleic Acid Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions.

Real-time PCR assays were done using the LightCycler real-time PCR machine 1.2 and 2.0 (Roche Diagnostics) and software versions 3.5, 4.0, and 4.05. For quantification analysis, the 2nd derivative maximum method with proportional baseline adjustment (software version 3.5) or absolute quantification with the automated F'' max method (software versions 4.0. and 4.05) was used. For conventional PCR assays, the PerkinElmer GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA) was used. All the PCR assays included several water samples as negative controls and either a positive control or a standard dilution series of 80000 to 8.0 GEs of *C. pneumoniae* DNA extracted from purified *C. pneumoniae* elementary bodies (strain Kajaani 7), and diluted with MS2 RNA solution (Roche Diagnostics). The positive controls and standards were always handled last. Care was taken to avoid contamination during DNA extraction and the PCR. Precautions included separate rooms, pipettes, gloves, and laboratory coats for DNA extraction, PCR mix pipeting, sample pipeting, PCR, and gel electrophoresis, frequent change of gloves during sample handling, use of UV light laminar flow hoods, aerosol-resistant pipette tips, and several negative controls. If contamination was observed, the results of the PCR assay were rejected, all laboratories and equipment were carefully cleaned, and reagents were replaced by unopened ones. The samples were tested for PCR inhibition by spiking some samples with 10.0 GEs of *C. pneumoniae* DNA. In
addition, parts of the samples that were PCR-negative when undiluted were also tested as 1:3 and 1:10 dilutions.

4.5.2 In situ hybridization (III)

Paraffin-embedded atherosclerotic tissue samples were obtained as described earlier. Five-micrometer-thick sections were cut and placed on Super Frost Plus slides (O. Kindler, Freiberg, Germany). The sections were attached at room temperature overnight, followed by +37 °C overnight, and stored at +4 °C until analysis. To detect *C. pneumoniae* DNA from atherosclerotic tissue samples, an ISH method modified from a previously published method (Alakärppä *et al.* 1999) was used. A DNA probe that detects the *C. pneumoniae*-specific region of the 16S rRNA gene was synthesized using a PCR DIG Probe Synthesis Kit (Roche Diagnostics). The bound probe was visualized with an alkaline phosphatase-conjugated sheep antidigoxigenin antibody (Roche Diagnostics) and Fast Red dye (Dako, Carpinteria, CA), and the sections were counterstained with Mayer’s hematoxylin (Signet Laboratories, Dedham, MA). Two sections per atherosclerotic tissue sample were studied. Each series included a negative control sample (hybridization without probe). To ensure the functionality of the ISH assay, we infected HL cells by centrifugation with one multiplicity of infection (2 × 10⁷ infection-forming units) of *C. pneumoniae* (strain Kajaani 7), and HL cells were grown for 72 hr under normal conditions. (The cell culture media contained cycloheximide). The cells were then detached by glass beads, pelleted, fixed with 10% formalin, and fused in paraffin. From the resulting block, 5-μm-thick sections were cut and treated similarly to the tissue samples.

4.5.3 Transmission electron microscopy (III)

The atherosclerotic tissue samples were prefixed in phosphate-buffered (pH 7.2) 2.5% glutaraldehyde for six hours, then washed with the same buffer, and stored in a refrigerator. Postfixation was performed with phosphate-buffered 1% osmium tetroxide for two hours. After dehydration with ethanol, embedding in epoxy resin, thin-sectioning with an ultramicrotome, and poststaining with uranyl acetate and lead citrate, the thin sections were examined using JEOL-1200EX operating at 60 kV. TEM was performed by Kari Lounatmaa (Oy Lounatmaa Ltd, FIN-00320 Helsinki, Finland).
4.5.4 Immunoelectron microscopy (III)

Fresh representative pieces of atherosclerotic tissue were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer with 2.5% sucrose, pH 7.4, for 2 hr. Smaller pieces of tissue were immersed in 2.3 mol/L sucrose and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT microtome. For immunolabeling, the sections were first incubated in 0.05 mol/L glycine in phosphate-buffered saline (PBS) followed by incubation in 5% bovine serum albumin (BSA) with 0.1% cold water fish skin gelatin (Aurion, Wageningen, The Netherlands) in PBS. Antibodies and gold conjugate were diluted in 0.1% BSA-C (Aurion) in PBS. All washings were performed in 0.1% BSA-C in PBS. The sections were then incubated with an antibody to cLPS (Anti-Chlamydia 6709 SP-5; Medix Biochemica, Kauniainen, Finland) for 60 min, followed by a secondary antibody to rabbit a-mouse IgG (Zymed, South San Francisco, CA) and protein A-gold complex [size, 10 nm; made after (Slot & Geuze 1985)] for 30 min. Controls were prepared by carrying out the labeling procedure without the primary antibody. The sections were embedded in methylcellulose and examined under a Philips CM100 transmission electron microscope (FEI, Eindhoven, The Netherlands). Images were captured by a CCD camera equipped with TCL-EMMenu version 3 from Tietz Video and Image Processing Systems (Gaunting, Germany). IEM was performed by Raija Sormunen in Biocenter Oulu and Department of Pathology, University of Oulu.

4.5.5 C. pneumoniae antibodies (II, IV)

MIF was used to measure serum C. pneumoniae IgG and IgA antibody levels, according to a previously published protocol (Wang 2000, Paldanius et al. 2003). EBs of C. pneumoniae strain Kajaani 6 (National Public Health Institute, Oulu, Finland) were used as an antigen. The sera were tested in serial fourfold dilutions to the end point. Three different positivity levels were applied to both the IgG and IgA antibody titres. For IgG antibodies, titres 32, 64, and 128 and for IgA antibodies, titres 10, 20, and 40 were used as limits for low-positive, positive and high-positive, respectively.
4.6 Detection of LPS (III, IV)

4.6.1 Detection of cLPS in tissue samples (III)

A 50-mg piece of atherosclerotic tissue was homogenized as previously described for DNA extraction, except that 400 μL of the kit’s (see later) transport medium was used as a homogenization buffer, and no proteinase K or overnight incubation was included. The homogenates were centrifuged at 18 330 × g for 10 min, and 200 μL of the supernatant was used for cLPS measurement with a commercial IDEIA Chlamydia kit, ref. no. K6002 (Dako, Cambridgeshire, UK), done with the standard assay procedure according to the manufacturer’s instructions, except that the initial treatment of samples and controls was performed at +37 °C for 30 min. Because the kit does not include standards, serial dilution of 0.1 to 430.0 ng/mL of purified cLPS (Tiirola et al. 2006), measured separately in triplicate, was used to generate a curve, to which the optical density values of the homogenized samples were compared.

4.6.2 Detection of cLPS and totLPS in serum samples (IV)

The serum levels of cLPS and total endotoxin activity (totLPS) were measured. Chlamydial LPS was measured from the sera using the LPS-binding protein-based method described earlier (Tiirola et al. 2006). In brief, serum cLPS was first solubilized with detergent (phosphate-buffered saline (PBS) containing 0.1% octylglucoside) and captured with LBP (1 μg/ml in 0.1% Tween 20-PBS). Complexes of LBP-cLPS were then bound to the solid phase by an anti-cLPS monoclonal antibody [MAb 6709 (Medix Biochemica Kauniainen, Finland) 5 μg/ml solution in PBS], and the bound complexes were detected with a polyclonal anti-LBP antibody (rabbit IgG antibodies against human LBP (HyCult Biotechnology) diluted 1:500 in 0.1% Tween 20–PBS), a peroxidase-labeled goat antirabbit antibody (GAR-HRP, IgG (H + L) antibody, Bio-Rad, Hercules, CA) and an HRP substrate (O-Phenylenediamide 0.4 mg/mL, (Sigma-Aldrich Chemie, Steinheim, Germany) prepared in 0.012% H2O2, 0.05 M Na2HPO4 ⋅ 2 H2O and 0.025 M citric acid-1-hydrate). Serum totLPS was determined with a kinetic Limulus Amebocyte Lysate (LAL) test kit with a chromogenic substrate (HyCult biotechnology b.v., Uden, Netherlands) on diluted (1:5, vol/vol in endotoxin-free water) samples. Measurement of totLPS was performed in the laboratory of Pirkko Pussinen in the Institute of Dentistry, University of Helsinki, and the
Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital.

4.7 Statistical analyses

In study II, Spearman’s and Pearson’s correlation, a Wald test, analysis of covariance, and linear regression were used. Tests for genetic associations were performed with an additive model for each polymorphism; tests were also performed for men and women separately. Multivariate analyses were performed only for the SNPs that showed a statistically significant association with the respective trait. For each model, 5000 permutations were run in order to control for multiple testing. In study III, the comparison of positivity rates between the different groups was done by Fisher’s exact test. In study IV, $\chi^2$ linear-by-linear association (for categorized variables) and a test for a trend across ordered groups (for continuous variables) were used to study the trends across the BMI groups, and analysis of covariance was used for multivariate analysis.

For the continuous variables that did not follow a normal distribution, a logarithmic transformation was done. If the distribution could not be normalized by any transformations, the continuous variables were used as categorized variables. The statistical analyses were done using SPSS for Windows 13.0 and 15.0, Stata 5.0 softwares (SPSS Inc. Chicago, Illinois, USA and Stata Corporation Inc., College Station, Texas, USA), and the analysis of polymorphisms in study II was done with PLINK for MS DOS v1.02. (Purcell et al. 2007) (URL: http://pngu.mgh.harvard.edu/purcell/plink/).
5 Results

5.1 Characteristics of the study populations

The characteristics of the four study groups (blood donors, CAR, AOD and AAA), and the combined CVD group are listed in Table 4. The Finnish Red Cross only gave information about the gender and age of the blood donors. Of the CVD group, only the CAR patients were included in study III, but study IV included all the patients with sufficient information for calculating their BMI (174 patients, 104 CAR, 21 AOD, and 49 AAA). Diabetes was classified as type I / II diabetes or no diabetes, smoking as current / during the last five years or non-smoking. Hypercholesterolemia was defined as measured high cholesterol values or statin treatment due to previous high values. Statin treatment and dosage were registered as yes or no at the time of admission; statin history was not registered systematically. Approximately half of the patients (55.2%, n = 96) had either a coronary artery disease or a previously registered arterial disease in addition to their cerebral or peripheral vascular disease. A majority of the patients (70.1%, n = 122) used salicylates. Use of non-steroidal anti-inflammatory drugs was likely infrequent since all patients with salicylate treatment due to atherosclerosis are advised against it. There were not many steroid users in the series.

Table 4. Characteristics of the blood donors and CVD patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>blood donors</th>
<th>CAR</th>
<th>AOD</th>
<th>AAA</th>
<th>CVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, median (IQR)</td>
<td>47.0</td>
<td>70.3</td>
<td>59.6</td>
<td>72.0</td>
<td>70.3</td>
</tr>
<tr>
<td></td>
<td>(36.5–55.0)</td>
<td>(60.1–74.1)</td>
<td>(51.0–70.4)</td>
<td>(63.5–77.3)</td>
<td>(60.2–74.6)</td>
</tr>
<tr>
<td>n men/ n women (% men)</td>
<td>271/122 (69)</td>
<td>80/30 (72.3)</td>
<td>12/10 (54.5)</td>
<td>45/5 (90)</td>
<td>137/45 (75.3)</td>
</tr>
<tr>
<td>Smoking, % (n)</td>
<td>NA</td>
<td>39.1 (43)</td>
<td>90.9 (20)</td>
<td>52.0 (26)</td>
<td>48.9 (89)</td>
</tr>
<tr>
<td>Statins, % (n)</td>
<td>NA</td>
<td>62.7 (69)</td>
<td>27.3 (6)</td>
<td>42.0 (21)</td>
<td>52.7 (96)</td>
</tr>
<tr>
<td>Hypercholesterolemia, % (n)</td>
<td>NA</td>
<td>71.8 (79)</td>
<td>40.9 (9)</td>
<td>52.0 (26)</td>
<td>62.6 (114)</td>
</tr>
<tr>
<td>Diabetes type I or II, % (n)</td>
<td>NA</td>
<td>22.7 (25)</td>
<td>18.2 (4)</td>
<td>16.0 (8)</td>
<td>20.3 (37)</td>
</tr>
<tr>
<td>BMI kg/m², mean (SD)</td>
<td>NA</td>
<td>26.5 (3.5)</td>
<td>26.6 (3.8)</td>
<td>26.5 (3.5)</td>
<td>26.5 (3.5)</td>
</tr>
</tbody>
</table>

All the patients were older than the blood donors, the Mann-Whitney test p-value was < 0.001 for all the comparisons between blood donors and CVD groups. In the gender distribution, the only statistically significant difference was between blood donors and AAA (the Fisher’s exact test p = 0.001). Among the CVD study population, statistically significant differences between the patient groups were
found in age, sex distribution, smoking, statins, and hypercholesterolemia. For the age comparison, the Mann-Whitney test p-values were 0.01 for CAR and AOD, 0.03 for CAR and AAA, and < 0.001 for AOD and AAA. For gender distribution, there was a difference between CAR and AAA (Fisher’s exact test p = 0.014) and between the AOD and AAA (p = 0.001). In smoking, differences were found between CAR and AOD (p < 0.001) and between AOD and AAA (p = 0.001). In hypercholesterolemia, a difference was found between CAR and AOD (p = 0.012), and between CAR and AAA (p=0.019). In statins, there was a difference between CAR and AOD (p = 0.004) and between CAR and AAA (p = 0.017).

Table 5 shows the serum levels of the measured inflammatory and innate immunity markers among the blood donors and both the combined and separate CVD study groups. All the patient groups and the combined CVD group had clearly higher serum IL-6, hsCRP, LBP, and sCD14 levels than the blood donor group did (Mann-Whitney test p-values < 0.001 for all but LBP between blood donors and CAR p = 0.008, and for sCD14 between blood donors and AOD p = 0.045). Between the CAR and AOD groups there was a statistically significant difference in IL-6 (p = 0.044), LBP (p = 0.012) and sCD14 (p = 0.048). Between the CAR and AAA groups, a difference was found in IL-6 (p < 0.001), CRP (p = 0.007) and in LBP (p = 0.004). Finally, between the AOD and AAA groups, there was a difference only in the sCD14 concentration (p = 0.020).

Table 5. Continuous variables among the blood donors and CVD patients.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Blood donors</th>
<th>CAR</th>
<th>AOD</th>
<th>AAA</th>
<th>CVD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 392</td>
<td>N = 110</td>
<td>N = 22</td>
<td>N = 50</td>
<td>N = 182</td>
</tr>
<tr>
<td>md (IQR)</td>
<td>md (IQR)</td>
<td>md (IQR)</td>
<td>md (IQR)</td>
<td>md (IQR)</td>
<td></td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>0.41 (0.01–1.02)</td>
<td>2.08 (0.74–3.06)</td>
<td>2.65 (1.73–4.10)</td>
<td>3.96 (2.70–5.50)</td>
<td>2.56 (1.25–4.17)</td>
</tr>
<tr>
<td>hsCRP, mg/l</td>
<td>0.58 (0.31–1.16)</td>
<td>1.40 (0.60–3.03)</td>
<td>2.50 (1.00–4.70)c</td>
<td>2.45 (1.08–7.65)</td>
<td>1.80 (0.75–4.70)c</td>
</tr>
<tr>
<td>sCD14, μg/ml</td>
<td>1.59 (1.27–1.94)c</td>
<td>1.95 (1.70–2.25)</td>
<td>1.72 (1.60–2.17)</td>
<td>2.01 (1.75–2.35)</td>
<td>1.94 (1.69–2.26)</td>
</tr>
</tbody>
</table>

C. pneumoniae antibody positivity by category in each study group and in the combined CVD group is shown in Table 6. A higher antibody positivity rate in all categories was seen in all the patient groups when compared with the blood donors. However, not all the differences reached statistical significance (Table 6) When the CVD study groups were compared with each other, a difference was only observed between the CAR and AAA groups, in the categories IgG ≥ 32 (Fisher’s exact test p = 0.002) and IgG ≥ 32 and/or IgA ≥ 10 (p = 0.003).
Table 6. *C. pneumoniae* antibody positivity. Shown as % (n) in the different categories in the study groups.

<table>
<thead>
<tr>
<th>Category</th>
<th>Blood donors</th>
<th>CAR</th>
<th>AOD</th>
<th>AAA</th>
<th>CVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG ≥ 32&lt;sup&gt;a, b, c, d, e&lt;/sup&gt;</td>
<td>40.8 (160)</td>
<td>60.9 (67)</td>
<td>63.6 (14)</td>
<td>86.0 (43)</td>
<td>68.1 (124)</td>
</tr>
<tr>
<td>IgG ≥ 64&lt;sup&gt;a, c, d&lt;/sup&gt;</td>
<td>13.0 (51)</td>
<td>31.8 (35)</td>
<td>27.3 (6)</td>
<td>44.0 (22)</td>
<td>34.6 (63)</td>
</tr>
<tr>
<td>IgA ≥ 10&lt;sup&gt;c, d&lt;/sup&gt;</td>
<td>19.1 (75)</td>
<td>26.4 (29)</td>
<td>22.7 (5)</td>
<td>34.0 (17)</td>
<td>28.0 (51)</td>
</tr>
<tr>
<td>IgA ≥ 20&lt;sup&gt;c, d&lt;/sup&gt;</td>
<td>7.9 (31)</td>
<td>15.5 (17)</td>
<td>9.1 (2)</td>
<td>20.0 (10)</td>
<td>15.9 (29)</td>
</tr>
<tr>
<td>IgG ≥ 32 and/or IgA ≥ 10&lt;sup&gt;c, d, e&lt;/sup&gt;</td>
<td>43.6 (171)</td>
<td>62.7 (69)</td>
<td>63.6 (14)</td>
<td>86.0 (43)</td>
<td>69.2 (126)</td>
</tr>
<tr>
<td>IgG ≥ 64 and/or IgG ≥ 20&lt;sup&gt;c, d&lt;/sup&gt;</td>
<td>15.6 (61)</td>
<td>35.5 (39)</td>
<td>27.3 (6)</td>
<td>46.0 (23)</td>
<td>37.4 (68)</td>
</tr>
</tbody>
</table>

Statistically significant difference in comparisons <sup>b</sup>blood donors→CAR, <sup>c</sup>blood donors→AOD, <sup>d</sup>blood donors→AAA, <sup>e</sup>blood donors→CVD, and <sup>f</sup>CAR→AAA.

5.2 LBP genotyping method (I)

Fig. 4 represents typical derivative melting curves of the three different LBP <sup>C</sup>1341→T (Leu436→Phe) polymorphism genotypes. The mean (SD) melting temperature of the T allele was 54.39 °C (0.72 °C) and that of the C allele was 60.78 °C (0.45 °C). The maximal variation in observed melting temperatures was within 3.74 °C for the T allele and within 2.80 °C for the C allele. For all the sequenced samples, the sequencing results were identical to the genotypes obtained with our PCR method.

The allele and genotype frequencies of the LBP polymorphism in the blood donor study group are presented in the context of the other genotyping results in Table 8.
5.3 Gene polymorphisms and serum levels of markers of inflammation, innate immunity, and C. pneumoniae infection (II)

5.3.1 Markers of inflammation and innate immunity

Among the blood donors, the serum levels of IL-6, hsCRP, LBP, and sCD14 were generally low and within the limits of a normal, healthy population (Table 5). Five persons had serum IL-6 > 20.0 pg/mL, six had hsCRP > 10.0 mg/L, and four had LBP > 35 μg/mL. These highest values were omitted from further statistical analysis since they might reflect an ongoing inflammatory reaction. The serum levels of sCD14 were within normal limits in the whole study population. The men had slightly higher serum levels of sCD14 (t-test p = 0.002) and IL-6 (Mann-
Whitney test \( p = 0.060 \) than the women, and they also tended to be slightly older than the women (Mann–Whitney test \( p = 0.075 \)). The mean (SD) concentration of serum was 1.65 \( \mu g/ml \) (0.56 \( \mu g/ml \)) for the men and 1.45 \( \mu g/ml \) (0.54 \( \mu g/ml \)) for the women. The median (IQR) concentration of serum IL-6 was 0.47 pg/ml (0.08–0.96 pg/ml) among the men, and 0.23 pg/ml (0.00–1.05 pg/ml) among the women. The median (IQR) age was 47.0 years (40.0–55.0 years) for the men and 45.5 years (29.0–55.0 years) for the women.

The Spearman’s correlations between age and the serum concentrations of IL-6, hsCRP, sCD14, and LBP are shown in Table 7. A positive correlation between age and IL-6, hsCRP, and LBP was observed. Serum IL-6 was positively correlated with hsCRP, and correlations with LBP and sCD14 were also observed. A positive correlation existed between hsCRP and LBP, and hsCRP was also correlated with sCD14. In addition, a positive correlation between LBP and sCD14 was seen. A majority of these correlations remained statistically significant in the separate groups of men and women.

Table 7. Correlations between age and the serum markers of inflammation and innate immunity in the blood donors.

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL-6, pg/ml</th>
<th>hsCRP, mg/l</th>
<th>LBP, ( \mu g/ml )</th>
<th>sCD14, ( \mu g/ml )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>0.106*</td>
<td>0.187***</td>
<td>0.100* a</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>–</td>
<td>0.397***</td>
<td>0.266***</td>
<td>0.184***</td>
</tr>
<tr>
<td>hsCRP, mg/l</td>
<td>–</td>
<td>–</td>
<td>0.351***</td>
<td>0.154**</td>
</tr>
<tr>
<td>LBP, ( \mu g/ml )</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.159** a</td>
</tr>
</tbody>
</table>

*Pearson’s correlation, *\( p < 0.05 \); **\( p < 0.01 \), ***\( p < 0.001 \)

### 5.3.2 Gene polymorphisms

Table 8 presents the genotype and allele frequencies of the IL-6, CD14, LBP, TLR-2, and TLR-4 gene polymorphisms in the blood donor study group. All the polymorphisms studied were in Hardy-Weinberg equilibrium. There was no statistically significant difference in the genotype distribution between the genders. The effect of the gene polymorphisms on the serum levels of sCD14 and LBP were assessed in study II. We also looked for associations with serum IL-6 and hsCRP and markers of C. pneumoniae infection.

The LBP C\(_{1341}\)→T (Leu\(_{436}\)→Phe) polymorphism and the TLR-4 A\(_{896}\)→G (Asp\(_{299}\)→Gly) polymorphism were associated with serum levels of LBP (Table 9). The effect of the LBP polymorphism was seen in the whole study population, but
the association was more distinct among the men (Table 9). The effect of the TLR-4 polymorphism again, was observed only among the women (Table 9). LBP concentration according to the different polymorphisms and genotypes are shown in Table 10. In multivariate analyses with age and sex as independent variables for the whole population and with age as an independent variable for men, the association between the LBP polymorphism and serum LBP remained statistically significant (Table 9). In addition, age was statistically significant in the whole population (p = 0.034). In a multivariate analysis for women, with age included as an independent variable, the association between the TLR-4 polymorphism and serum LBP remained statistically significant (Table 9). In addition, higher age was associated with an increase in serum LBP concentration (p = 0.020). We also studied if the effects of these polymorphisms are independent from each other. For this analysis both of the polymorphisms, age, and gender (for the whole population) were included in the model as independent variables. The effects of the polymorphisms were found to be independent in the whole study population, among the men, and among the women (Table 9). In these analyses, age was statistically significant in the whole study population (p = 0.032) and among the women (p = 0.020).

Table 8. Genotype and allele distribution in the blood donors. Shown as % (n), the first row for each polymorphism shows the number of persons genotyped.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>IL-6 G−174→C</th>
<th>LBP C1341→T</th>
<th>CD14 C−260→T</th>
<th>TLR-4 A896→G</th>
<th>TLR-2 G2408→A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alleles</td>
<td>G/C</td>
<td>C/T</td>
<td>C/T</td>
<td>A/G</td>
<td>G/A</td>
</tr>
<tr>
<td>Homozygote 1*</td>
<td>30.0 (117)</td>
<td>86.0 (338)</td>
<td>43.3 (170)</td>
<td>80.7 (317)</td>
<td>94.4 (371)</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>51.0 (199)</td>
<td>13.0 (51)</td>
<td>42.5 (167)</td>
<td>18.1 (71)</td>
<td>5.3 (21)</td>
</tr>
<tr>
<td>Homozygote 2</td>
<td>19.0 (74)</td>
<td>1.0 (4)</td>
<td>14.2 (56)</td>
<td>1.3 (5)</td>
<td>0.3 (1)</td>
</tr>
<tr>
<td>Minor allele frequency</td>
<td>0.44</td>
<td>0.08</td>
<td>0.35</td>
<td>0.10</td>
<td>0.03</td>
</tr>
</tbody>
</table>

|                | G for IL-6, C for LBP, CC for CD14, AA for TLR-4, and GG for TLR-2 |

Among the men, a trend for association of the CD14 C−260→T polymorphism with sCD14 concentration was observed; the increase in sCD14 concentration per each copy of the T allele was 0.12 μg/ml (95% CI = 0.07–0.17 μg/ml), p = 0.042, and the corrected p = 0.196. The serum sCD14 concentration according to the CD14 genotypes is shown in Table 10. In a multivariate analysis for men, with
age included as an independent variable, the increase in sCD14 concentration was 0.11 μg/ml (0.003–0.214 μg/ml) for each copy of the T allele, p = 0.045 and the corrected p = 0.206.

Table 9. Effect of LBP and TLR-4 gene polymorphisms on serum concentration of LBP in the blood donors.

<table>
<thead>
<tr>
<th>Group</th>
<th>Polymorphism</th>
<th>Allele 1</th>
<th>Mean change (95% CI)</th>
<th>P-value</th>
<th>Corrected p-value</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>LBP C1341→T</td>
<td>C</td>
<td>2.21 (1.49–2.93)</td>
<td>0.002</td>
<td>0.047</td>
<td>unadjusted</td>
</tr>
<tr>
<td>All</td>
<td>LBP C1341→T</td>
<td>C</td>
<td>2.25 (0.84–3.66)</td>
<td>0.002</td>
<td>0.010</td>
<td>sex and age</td>
</tr>
<tr>
<td>Men</td>
<td>LBP C1341→T</td>
<td>C</td>
<td>2.74 (1.89–3.59)</td>
<td>0.001</td>
<td>0.009</td>
<td>unadjusted</td>
</tr>
<tr>
<td>Men</td>
<td>LBP C1341→T</td>
<td>C</td>
<td>2.73 (1.07–4.39)</td>
<td>0.001</td>
<td>0.009</td>
<td>age</td>
</tr>
<tr>
<td>Women</td>
<td>TLR-4 A896→G</td>
<td>G</td>
<td>−3.00 (−4.12…−1.88)</td>
<td>0.008</td>
<td>0.045</td>
<td>unadjusted</td>
</tr>
<tr>
<td>Women</td>
<td>TLR-4 A896→G</td>
<td>G</td>
<td>−3.07 (−5.21…−0.92)</td>
<td>0.006</td>
<td>0.030</td>
<td>age</td>
</tr>
<tr>
<td>All</td>
<td>LBP C1341→T</td>
<td>C</td>
<td>2.23 (0.82–3.64)</td>
<td>0.002</td>
<td>0.002</td>
<td>sex, age and TLR-4 A896→G</td>
</tr>
<tr>
<td>Men</td>
<td>LBP C1341→T</td>
<td>C</td>
<td>2.73 (1.06–4.39)</td>
<td>0.001</td>
<td>0.001</td>
<td>age and TLR-4 A896→G</td>
</tr>
<tr>
<td>Women</td>
<td>TLR-4 A896→G</td>
<td>G</td>
<td>−2.97 (−5.13…−0.82)</td>
<td>0.008</td>
<td>0.009</td>
<td>age and LBP C1341→T</td>
</tr>
</tbody>
</table>

1 Change per one copy of allele 1

Table 10. Levels of serum markers according to the gene polymorphisms in the blood donors.

<table>
<thead>
<tr>
<th>Group</th>
<th>Polymorphism</th>
<th>Genotype (n)</th>
<th>Serum marker Name</th>
<th>Serum marker Mean (Std. deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>LBP C1341→T</td>
<td>CC (4)</td>
<td>LBP, μg/ml</td>
<td>10.28 (7.14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT (51)</td>
<td></td>
<td>13.34 (5.49)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT (338)</td>
<td></td>
<td>10.28 (5.33)</td>
</tr>
<tr>
<td>Men</td>
<td>LBP C1341→T</td>
<td>CC (2)</td>
<td>LBP, μg/ml</td>
<td>14.39 (13.73)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT (35)</td>
<td></td>
<td>13.19 (5.84)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT (234)</td>
<td></td>
<td>10.26 (4.93)</td>
</tr>
<tr>
<td>Women</td>
<td>TLR-4 A896→G</td>
<td>GG (3)</td>
<td>LBP, μg/ml</td>
<td>4.30 (3.49)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG (25)</td>
<td></td>
<td>8.79 (6.41)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA (94)</td>
<td></td>
<td>11.43 (5.88)</td>
</tr>
<tr>
<td>Men</td>
<td>CD14 C260→T</td>
<td>CC (125)</td>
<td>sCD14, μg/ml</td>
<td>1.57 (0.49)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT (113)</td>
<td></td>
<td>1.72 (0.56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT (33)</td>
<td></td>
<td>1.67 (0.79)</td>
</tr>
</tbody>
</table>
The only polymorphism associated with *C. pneumoniae* antibody positivity was the *IL-6* G−174→C polymorphism. Those with IgG ≥ 64 and/or IgA ≥ 20 had a G allele frequency of 56.56%, whereas those with lower antibody positivity had a frequency of 42.38%, p = 0.004, and corrected p = 0.025. In a multivariate analysis with age and sex as covariates, the G allele OR (95% CI) was 1.83 (1.22–2.74). Age and sex were not statistically significant.

The *TLR2* G2408 →A (Arg753 → Gln) polymorphism was not associated with any of the studied serum inflammation and innate immunity markers or *C. pneumoniae* antibodies. For serum hsCRP and IL-6 no association with any of the polymorphisms was found.

5.4 Comparison of PCR methods, in situ hybridization, and EIA for detection of *C. pneumoniae* in atherosclerotic carotid plaques (III)

5.4.1 PCR experiments for detection of *C. pneumoniae*

The actual experiments were combinations of the DNA extractions and PCR assays described in the methods section. The PCR experiments were carried out during the years 2003–2006, and at the time of the first experiments, not all of the samples had been collected yet, and for the last experiments, there was no tissue left from some of the samples. Of the available samples, a subset for each experiment was chosen randomly, thus not all of the samples were tested in all the experiments. The experiments differed in the amount of tissue used, in the storage of DNA, and in the PCR assay (Table 11). Table 12 shows the results of the different PCR experiments.

Table 11. Settings of the *Chlamydia pneumoniae* PCR experiments.

<table>
<thead>
<tr>
<th>PCR assay</th>
<th>DNA storage (°C)</th>
<th>Sample material</th>
<th>No. of patients</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semiconventional Pst PCR</td>
<td>−20</td>
<td>50-mg tissue</td>
<td>63</td>
<td>1A</td>
</tr>
<tr>
<td></td>
<td>−20</td>
<td>5 x 5-mg tissue</td>
<td>99</td>
<td>1B</td>
</tr>
<tr>
<td></td>
<td>+4</td>
<td>5 x 5-mg tissue</td>
<td>22</td>
<td>1C</td>
</tr>
<tr>
<td>16S LC-PCR</td>
<td>+4</td>
<td>5 x 5-mg tissue</td>
<td>58</td>
<td>2</td>
</tr>
<tr>
<td>16S LC-PCR and conventional Pst PCR</td>
<td>−20</td>
<td>2 or 4 5-μm-thick sections</td>
<td>30</td>
<td>3</td>
</tr>
</tbody>
</table>

*PCR was performed on the day after extraction*

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Table 12. Results of the \textit{C. pneumoniae} PCR experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of double positives/no. of positives (no. of patients) in both experiments</th>
<th>No. of positives/no. of patients (% positive) in each experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1/4 (60) &lt; 0.001 1/8 (15) &lt; 0.001 NS 0/1 (11) NS 3/63 (4.8)</td>
<td>NS &lt; 0.001 &lt; 0.001 NS 8/99 (8.1)</td>
</tr>
<tr>
<td>1B</td>
<td>– 2/12 (22) &lt; 0.001 1/19 (58) &lt; 0.001 NS 0/16 (41) NS 0/1 (11) NS 8/99 (8.1)</td>
<td></td>
</tr>
<tr>
<td>1C</td>
<td>– – 3/8 (12) NS 0/1 (3) 0.013 0/7 (23) NS 0.013 10/22 (45.5)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>– – – 0/2 (10) NS 0/16 (41) NS 0/1 (11) NS 8/99 (8.1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>– – – – 4/30 (13.3)</td>
<td></td>
</tr>
</tbody>
</table>

\( P \) values shown are for Fisher’s exact test for comparison of positivity rate of each of the two PCR experiments.

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In experiments (1B, 1C, and 2) where five 5-mg pieces of atherosclerotic tissue were used, the plaque was considered PCR-positive if one of the pieces was PCR-positive. There were 10 plaques with multiple (2–5) PCR-positive 5-mg pieces, and most of them were in PCR experiment 2, which used quantitative PCR. The quantification results varied from < 0.1 to > 200 GEs/mg tissue, the median (IQR) being 8.9 GEs/mg tissue (3.4–12.6 GEs/mg). The maximum observed variation in the \textit{C. pneumoniae} load between different pieces of a given plaque was 3.3 to 204.2 GEs/mg tissue.

When the positivity rates of the different PCR experiments were compared, the highest PCR positivity rates were observed in experiments 1C and 2, which used freshly extracted DNA from 5 × 5-mg samples (Table 12). In a combination of any two PCR experiments, the maximum number of patients that had a positive PCR result in both of the experiments was three (Table 12). For each patient, one to five PCR experiments were performed and the overall PCR positivity rate of the different PCR experiments was 32.4% (35 positives/108 patients).

### 5.4.2 Quality controls for \textit{C. pneumoniae} PCRs

In the whole study, 61 DNA samples were PCR-positive and 45 of them could be retested by conventional Pst PCR. Only 9 (20.0%) of the DNA samples were positive in retesting.
Some inhibition was observed in the quantitative 16S LC-PCR. All of the spiked (10 GEs) samples were positive, but in 9.8% (n = 5/55) of the samples < 7.0 GEs were recovered. Inhibition was observed in 13.3% (4/30) of the 50-mg samples and in 4.8% (1/21) of the 5-mg samples. Not enough DNA was available for inhibition tests of the paraffin-embedded samples. No inhibition was seen when these samples were retested with a conventional Pst PCR or semiconventional Pst PCR.

Of the \textit{C. pneumoniae} PCR-negative DNA samples, 143 (from 68 patients) were randomly selected to be tested as 1:3 and 1:10 dilutions by 16S LC-PCR. In these tests, eight (5.6%) DNA samples, all from 5-mg pieces and five different patients, were positive. Altogether, two previously totally PCR-negative patients became positive when diluted DNA was tested.

\section*{5.4.3 \textit{C. pneumoniae} ISH}

The functionality of \textit{C. pneumoniae} ISH was tested with \textit{C. pneumoniae}-infected cell culture samples. In these, positively labeled \textit{C. pneumoniae} inclusions could easily be seen inside the cells. However, handling and interpretation of the plaque samples was complicated by technical problems, like torn or partially washed away sections and some unspecific staining. Thus, only clear staining that was near or inside a cell was considered a positive label. In addition, only the patients with positive staining in both plaque sections were considered \textit{C. pneumoniae} ISH-positive. With these criteria, 39.4% (41/104) of the plaques were ISH-positive.

\section*{5.4.4 EIA for detection of cLPS in atherosclerotic plaques}

Plaque samples of 81 patients were examined with the commercial cLPS IDEIA test (Dako). The positivity limit was as recommended by the kit manufacturer (≥ 0.05 absorbance units higher than the mean of the absorbance values of the negative controls). With this positivity limit, 22.2% (n = 18) of the patients were cLPS-positive. When the optical density (OD) values of the samples were compared with those from serial dilutions of purified cLPS, the positivity limit corresponded to 4.7 pg cLPS/mg of tissue, and the highest observed OD value corresponded to 67.9 pg cLPS/mg of tissue.
5.4.5 TEM and IEM for C. pneumoniae

Careful examination with TEM revealed chlamydia-like particles (Fig. 5A) in the plaque samples of all of the patients. No other bacteria were seen. To verify these findings, IEM was performed on the plaque samples of three patients. Positive labeling for cLPS was seen in the cell membranes in all three plaques, and chlamydia-like particles were seen in one of the plaques (Fig. 5B). In the IDEIA test all these plaques had a low cLPS level and were not positive. However, all the plaques tested by IEM were positive both in PCR and in ISH.

Fig. 5. EM and IEM findings. (A) An electron micrograph presenting chlamydia-like particles (arrows) in an atherosclerotic tissue sample. The bar size is 200 nm. (B) An immunoelectron micrograph from the same sample showing chlamydia-like particles labeled with cLPS-specific antibody. The bar size is 500 nm. Reprinted from Lajunen et al. (2008) with permission from Elsevier.

5.4.6 Comparison of different C. pneumoniae detection methods

The distribution of positive results between the different methods (PCR, ISH, and cLPS EIA) is shown in Table 13. The concordance between any two markers was around 14%. The positivity rate was higher in the ISH and PCRs than in the cLPS measurement, but only the difference between the ISH and cLPS was statistically significant (Fisher’s exact test p = 0.017). Altogether 43 (39.1%) of the 110 patients
were negative for plaque *C. pneumoniae* markers. No sensitivity and specificity values were calculated because none of the methods used could be considered as the gold standard assay. There was no statistically significant association between age, sex, or smoking and PCR, ISH, or cLPS positivity.

**Table 13. Positive results in the different *Chlamydia* detection methods.**

<table>
<thead>
<tr>
<th>Method</th>
<th>% (no.) of patients positive with one method</th>
<th>% (no.) of patients positive with all methods</th>
<th>Total % (no.) of patients positive</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque cLPS</td>
<td>22.2 (18) NA</td>
<td>NA</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>ISH</td>
<td>39.4 (41) NA</td>
<td>NA</td>
<td>104</td>
<td>104</td>
</tr>
<tr>
<td>Any PCR&lt;sup&gt;1&lt;/sup&gt;</td>
<td>34.3 (37) NA</td>
<td>NA</td>
<td>108</td>
<td>108</td>
</tr>
<tr>
<td>cLPS and PCR</td>
<td>30.9 (25) 13.6 (11)</td>
<td>44.4 (36)</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>cLPS and ISH</td>
<td>36.0 (27) 14.7 (11)</td>
<td>50.7 (38)</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>PCR&lt;sup&gt;1&lt;/sup&gt; and ISH</td>
<td>46.1 (47) 13.7 (14)</td>
<td>59.8 (61)</td>
<td>102</td>
<td>102</td>
</tr>
<tr>
<td>PCR&lt;sup&gt;1&lt;/sup&gt; and/or ISH</td>
<td>NA</td>
<td>60.2 (62)</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>All three methods</td>
<td>38.7 (29) 9.3 (7)</td>
<td>65.3 (49)</td>
<td>75</td>
<td>75</td>
</tr>
</tbody>
</table>

Only the patients that were tested with all the listed methods are included in each row.

<sup>1</sup>Includes the samples positive as 1:3 or 1:10 dilutions.

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### 5.5 Association of serum cLPS and hsCRP levels with an elevated BMI in patients with CVD (IV)

The associations between chlamydial infection markers (cLPS and antibodies), totLPS, hsCRP, and BMI were examined. Serum cLPS correlated with hsCRP, and a weaker positive correlation between BMI and serum cLPS and hsCRP concentrations was also observed (Table 14).

**Table 14. Correlations between the continuous variables in the CVD patients.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>cLPS, ng/mL</th>
<th>hsCRP, mg/L</th>
<th>HDL, mmol/L</th>
<th>TG, mmol/L</th>
<th>LDL, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>NS</td>
<td>NS</td>
<td>0.277 **</td>
<td>−0.340 **</td>
<td>NS</td>
</tr>
<tr>
<td>BMI</td>
<td>0.197 **</td>
<td>0.195 **</td>
<td>−0.184 **</td>
<td>0.167 *</td>
<td>NS</td>
</tr>
<tr>
<td>totLPS, EU/mL</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.176 *</td>
<td>NS</td>
</tr>
<tr>
<td>cLPS, ng/mL</td>
<td>−</td>
<td>0.499 **</td>
<td>NS</td>
<td>NS</td>
<td>0.190 *</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>−</td>
<td>−</td>
<td>NS</td>
<td>0.090 *</td>
<td>0.151 *</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−0.364 **</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pearson correlation for BMI and cLPS, *p < 0.05; **p < 0.01.
Analysis of covariance was used to further study the factors associated with serum cLPS and hsCRP concentrations. To analyze cLPS, disease group, gender, age, smoking, diabetes, BMI, and HDL, LDL and TG levels were included in the model as independent variables. The variables that were not statistically significant (disease group, smoking, gender, and TG and HDL levels) were excluded from the final model. A higher serum cLPS concentration was associated with higher LDL (p = 0.004), higher BMI (p = 0.037), and higher age (p = 0.015). In addition, the association between diabetes and higher cLPS approached statistical significance (p = 0.099). The adjusted mean cLPS concentration (95% CI) for those 75 years or older 155.2 ng/ml (132.7–177.7 ng/ml) and for those younger than 75 years, it was 125.0 ng/ml (110.5–139.4 ng/ml). For the diabetic patients the respective values were 151.3 ng/ml (126.7–175.8 ng/ml) and for the non-diabetic patients, 128.9 ng/ml (115.7–148.1 ng/ml).

To analyze hsCRP, disease group, gender, age, smoking, BMI, use of statins, C. pneumoniae IgG positivity (≥ 64), C. pneumoniae IgA positivity (≥ 40), and cLPS, HDL, LDL, and TG levels were included in the model as independent variables. The variables that were not statistically significant (use of statins, age, C. pneumoniae IgA positivity, gender, disease group, and levels of HDL, LDL, and TG) were excluded from the final model. A higher serum hsCRP concentration was associated with a higher cLPS concentration (p < 0.001) and with C. pneumoniae IgG positivity (p = 0.013). In addition, the association between higher hsCRP and a higher BMI (p = 0.156), and smoking (p = 0.061) approached statistical significance. The adjusted geometric mean hsCRP concentration (95% CI) for those with C. pneumoniae IgG ≥ 64 was 2.64 mg/l (1.92–3.64 mg/l) and for those with IgG < 64 it was 1.59 mg/l (1.26–2.01 mg/l). For the smokers the respective values were 2.45 mg/l (1.86–3.25 mg/l) and for the non-smokers, 1.71 mg/l (1.31–2.23 mg/l).

To study the association between BMI and the other factors, the data were divided into three BMI groups: normal weight (BMI ≤ 24.9), overweight (BMI = 25.0–29.9), and obese (BMI ≥ 30.0). There were two persons with a BMI < 18.5, and they were included in the normal weight group. No statistically significant trend in the distribution of gender, disease group, smoking, statin use, diabetes, hypercholesterolemia, age, C. pneumoniae IgA antibody positivity, totLPS, LDL, or TG across the BMI groups was seen. The distribution of C. pneumoniae antibody positivity, cLPS, totLPS, hsCRP, HDL, LDL, and TG in the whole study population, across the BMI groups, and the p-value for trend, are shown Table 15.
Table 15. Characteristics of the CVD patients according to the BMI groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>All</th>
<th>BMI &lt; 25</th>
<th>BMI 25–29</th>
<th>BMI ≥ 30</th>
<th>p^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>174</td>
<td>65</td>
<td>80</td>
<td>29</td>
<td>NA</td>
</tr>
<tr>
<td>C. pneumoniae IgG:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 32, %</td>
<td>69.0</td>
<td>61.5</td>
<td>72.5</td>
<td>75.9</td>
<td>NS</td>
</tr>
<tr>
<td>≥ 64, %</td>
<td>35.1</td>
<td>24.6</td>
<td>38.8</td>
<td>48.3</td>
<td>0.018</td>
</tr>
<tr>
<td>≥ 128, %</td>
<td>29.9</td>
<td>20.0</td>
<td>33.8</td>
<td>41.4</td>
<td>0.026</td>
</tr>
<tr>
<td>C. pneumoniae IgA:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 10, %</td>
<td>28.7</td>
<td>30.8</td>
<td>25.0</td>
<td>34.5</td>
<td>NS</td>
</tr>
<tr>
<td>≥ 20, %</td>
<td>16.7</td>
<td>20.0</td>
<td>11.3</td>
<td>24.1</td>
<td>NS</td>
</tr>
<tr>
<td>≥ 40, %</td>
<td>14.9</td>
<td>16.9</td>
<td>10.0</td>
<td>21.1</td>
<td>NS</td>
</tr>
<tr>
<td>cLPS ng/ml, md (IQR)</td>
<td>123.3</td>
<td>92.6</td>
<td>128.9</td>
<td>146.4</td>
<td>0.01</td>
</tr>
<tr>
<td>totLPS EU/ml, md (IQR)</td>
<td>(72.3–167.9)</td>
<td>(50.8–167.0)</td>
<td>(76.4–163.9)</td>
<td>(105.8–175.8)</td>
<td>NS</td>
</tr>
<tr>
<td>hsCRP mg/l, md (IQR)</td>
<td>22.7 (14.9–60.5)</td>
<td>22.0 (16.1–58.1)</td>
<td>24.8 (14.3–59.5)</td>
<td>37.0 (15.6–69.0)</td>
<td>NS</td>
</tr>
<tr>
<td>HDL mmol/l, md (IQR)</td>
<td>1.90 (0.80–4.75)</td>
<td>1.70 (0.70–3.05)</td>
<td>1.70 (0.80–5.20)</td>
<td>3.40 (1.45–8.55)</td>
<td>0.01</td>
</tr>
<tr>
<td>LDL mmol/l, md (IQR)</td>
<td>1.10 (0.90–1.30)</td>
<td>1.16 (0.91–1.37)</td>
<td>1.03 (0.91–1.21)</td>
<td>1.06 (0.85–1.33)</td>
<td>0.06</td>
</tr>
<tr>
<td>TG mmol/l, md (IQR)</td>
<td>2.23 (1.82–2.79)</td>
<td>2.19 (1.68–2.77)</td>
<td>2.16 (1.86–2.79)</td>
<td>2.28 (1.96–2.84)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Analysis of covariance was used for a multivariate analysis of BMI. Disease group, gender, age, smoking, use of statins, hypercholesterolemia, diabetes, C. pneumoniae IgG positivity (≥ 64), and levels of totLPS, cLPS, hsCRP, HDL, LDL, and TG were included in the model as independent variables. Again, the variables that were not statistically significant (disease group, age, LDL, totLPS, gender, hsCRP, diabetes, use of statins, and hypercholesterolemia) were omitted from the final model. A higher BMI was associated with a higher cLPS concentration (p = 0.014), higher triglycerides (p = 0.017), C. pneumoniae IgG positivity (p = 0.025), and smoking (p = 0.029). In addition, the association between a higher BMI and lower HDL approached statistical significance (p = 0.060). For those with negative/low-positive C. pneumoniae IgG, the adjusted mean BMI (95% CI) was 26.1 m^2 (25.5–26.7 kg/m^2) and for those with relatively high IgG (≥ 64) it was 27.3 kg/m^2 (26.4–28.1 kg/m^2). The non-smokers had an adjusted mean BMI (95% CI) of 27.2 kg/m^2 (26.5–27.9 kg/m^2) and the smokers 26.1 kg/m^2 (25.4–26.9 kg/m^2).
6 Discussion

6.1 Study populations

In principle, all the studies in this work were cross-sectional, case-control studies. However, due to several reasons explained later in this chapter, in this work only either controls or cases were used in each study. This type of study setting does not affect interpretation of the results of methodological studies I and III. However, in study III healthy vascular tissue as a control would have been desirable, but due to Finnish legislation this was not possible. In studies II and IV the limitation due to the study design is that no conclusions can be made about the causality of the observed associations.

Study I was a methodological study in which the functionality of the genotyping method was tested on the blood donor samples, and the allele and genotype frequencies of this relatively healthy Finnish population were assessed.

In study II we were interested in the effect of the gene polymorphisms on the serum levels of markers of innate immunity. In addition, we studied the effect of the polymorphisms on markers of inflammation and \textit{C. pneumoniae} infection. Only the blood donor group was used for these studies, since the sample size in the CVD study groups is small and the serum levels of IL-6, hsCRP, LBP, and sCD14 are likely affected e.g. by the inflammatory state and medication of the patients. Thus, in the CVD study groups, the statistical power for finding associations for gene polymorphisms that have a small effect is low. On the other hand, in order to donate blood, a person must be generally healthy and at least two weeks must have passed since the end of symptoms of and/or medication for e.g. a feverish cold. This was reflected in the mainly low serum levels of inflammation and innate immunity markers in the blood donor group. Thus, we think this is a good study population for analyzing associations between gene polymorphisms and serum marker proteins. No comparison of the blood donors and the CVD study groups was done, since the patient groups were collected in the Helsinki area and the blood donors were from the Oulu region. As a result, the two groups may not represent the same background population, and thus a comparison of these two groups might be misleading.

In study III we were interested in the different plaque markers of \textit{C. pneumoniae} infection, and for the clarity of the study only the biggest CVD
The patient group (CAR) was included. Obviously, this could not be studied in the blood donor group, where no vascular tissue samples were available.

Finally, the blood donor group could not be included in study IV since there were no BMI values available for them.

As perhaps expected, the CVD patients were older, and had higher serum levels of inflammatory and innate-immunity markers and *C. pneumoniae* antibodies than the blood donors.

### 6.2 Methods

#### 6.2.1 DNA extraction (I, II, III)

Prior to DNA extraction, all the vascular tissue samples were homogenized in order to increase the DNA yield (III). The QIAamp DNA MiniKit used for DNA extraction has been reported to perform well with different starting materials and with material containing chlamydial DNA (Berg *et al.* 2003, Castella *et al.* 2006). The QIAamp DNA micro kit (QIAGEN) used for the paraffin-embedded tissue sections is again, specifically designed for DNA extraction from small amounts of starting material. To eliminate contaminations during DNA extraction, negative controls were included in all the extractions and tested in a PCR to be contamination-free. If any contamination was observed, all the laboratories and equipment were thoroughly cleaned and the samples were re-extracted.

#### 6.2.2 Genotyping methods (I, II)

A new method for genotyping the *LBP* C<sub>1341</sub>→T (Leu<sub>436</sub>→Phe) polymorphism was developed (I). The advantage of this new method when compared with the PCR-RFLP method used in previous studies (Hubacek *et al.* 2001, Hubacek *et al.* 2002) is that it is more rapid and easily interpretable. Distinguishing between the two alleles was easy, since the average difference in melting temperatures was 6.40 °C. All the sequenced samples confirmed the genotyping results of our real-time PCR-based method. All the other genotyping PCRs (II) [IL-6 G<sub>−174</sub>→C, CD14 C<sub>−260</sub>→T, TLR-4 A<sub>896</sub>→G (Asp<sub>299</sub>→Gly), and TLR-2 G<sub>2408</sub>→A (Arg<sub>753</sub>→Gln)] used previously published and verified real-time PCR methods (Heesen *et al.* 2000, Bertsch *et al.* 2001, Heesen *et al.* 2001, Heesen *et al.* 2003, Hamann *et al.* 2004). To obtain clearer results, some of the methods were slightly
modified for our use; practice has shown that these minor modifications are often needed when setting up a new real-time PCR method in a laboratory. Interpretation of all the results was clear, and a majority of the results were independently read by two persons. None of the polymorphisms studied showed deviation from Hardy-Weinberg equilibrium (HWE), indicating that systematic genotyping errors are unlikely (Lunetta 2008). In addition, the genotype and allele frequencies of the polymorphisms were similar to those previously observed in healthy persons/controls in Finnish populations (Järveläinen et al. 2001, Wang et al. 2001, Karhukorpi et al. 2002, Lorenz et al. 2002a, Veres et al. 2002) or in populations of European origin (Folwaczny et al. 2004).

6.2.3 C. pneumoniae PCR (III)

Three different PCR methods were used to detect of C. pneumoniae DNA in atherosclerotic tissue. In the literature, the positivity rates of C. pneumoniae PCRs for atherosclerotic tissue have varied greatly (Juvonen et al. 1997, Daus et al. 1998, Jantos et al. 1999, Ciervo et al. 2003, Mygind et al. 2003, Kozarov et al. 2006), possibly because of the use of different PCR methods, contamination, inhibition, and differencies in sample handling and the amount of tissue used (Apfalter et al. 2002, Apfalter et al. 2003). In our study, we observed great variation in the positivity rates (4.8% to 45.5%) between the different PCR experiments. In addition, the repeatability of positive results between the different experiments was poor, with a maximum of three patients positive in both of any two experiments. This clearly demonstrates that intralaboratory variation in PCR positivity may also be great. The observed variation may have been due to patchy distribution of C. pneumoniae DNA in the plaques, as shown by the variation in positivity and the C. pneumoniae load between the different 5-mg pieces of the same plaque, and also demonstrated by others (Smieja et al. 2001, Cochrane et al. 2003). In addition, the low copy number of C. pneumoniae DNA in the samples (the median value in our study was only ~9 copies per mg of tissue) is likely to play a role. In this situation, the effect of repeated freeze-thaw cycles on the PCR results may also be more important than in samples containing more template DNA (Bellete et al. 2003). This was reflected by the observations that in the PCR experiments where DNA was frozen and thawed prior to the PCR, the positivity rate was clearly lower when compared with freshly extracted DNA, and that positive results had poor repeatability. However, it is also possible that some false negative PCR results occurred due to inhibition, especially in the quantitative 16S
LC-PCR. Generally, the PCRs used in this study are sensitive, since they are capable of detecting the lowest positive control of 8 or 10 GEs (quantitative/semiquantitative and conventional PCRs, respectively) in every run.

6.2.4 C. pneumoniae ISH, TEM, and IEM (III)

With C. pneumoniae ISH, inclusions were detected inside the cells in the infected cell culture samples. However, use of the method with the plaque samples was complicated by technical problems. To reduce the possibility of false positive results, only samples where staining was observed near cells and staining was positive in both sections were regarded as positive. The positivity rate in our study was 39.4%, which is in agreement with our previous study, where 37.5% of atherosclerotic carotid artery specimens were C. pneumoniae ISH-positive (Mosorin et al. 2000). Generally, the ISH method was rather time-consuming and laborious to both perform and read.

Of the different C. pneumoniae detection methods, the highest positivity rate was yielded by EM, which revealed chlamydia-like particles in all samples after careful examination. Since the structures originating from degraded cells and vesicle-forming membranes can resemble chlamydia particles in EM, the samples were also studied with more specific methods. Some tissue samples were also tested for localization of cLPS with immunoelectronmicroscopy. In all these samples, cLPS associated with cell membranes was demonstrated, and chlamydia-like particles were seen in one sample. However, the tissue samples tested with IEM were not positive in cLPS EIA, even though all of them were positive in PCR, ISH, and EM. This difference may have been due to the uneven distribution of C. pneumoniae in the lesions. It is also likely that IEM is a more sensitive method than EIA, and even small amounts of cLPS can be detected with it. All the negative IEM controls were clean.

6.2.5 Detection of LPS (III, IV)

Atherosclerotic lesions (III)

Possible sources of error in the cLPS EIA method include cross-reactivity with cellular components and other bacteria. For instance, it has been reported that the locations of autofluorescent ceroid (a complex formed of oxidized lipid and
protein) deposits precisely match the areas that are immunoreactive for *C. pneumoniae*. However, direct reactivity of anti-*C. pneumoniae* antibodies with ceroid was not shown (Hoymans *et al.* 2004). Co-localization of ceroid and *C. pneumoniae* in atherosclerotic lesions could also be due to *C. pneumoniae*-induced LDL oxidation and macrophage foam cell formation, both of which processes are also associated with ceroid accumulation (Kalayoglu & Byrne 1998b, Kalayoglu & Byrne 1998a, Kalayoglu *et al.* 1999, Shor & Phillips 2000, Takahashi *et al.* 2002). In addition, the cLPS EIA kit is targeted for use on human samples, and the monoclonal antibodies used to detect cLPS react with KDO di- or trisaccharide moiety, which is not found in animal or human structures (Royo *et al.* 2000). The IDEIA Chlamydia kit has been tested to be non-reactive with 43 microbial genera and species (DakoCytomation 2003). However, this list does not include some Gram-negative organisms that may also be found in atherosclerotic lesions (Ameriso *et al.* 2001, Kowalski *et al.* 2002, Kozarov *et al.* 2006). Due to the structural differences of LPSs, it is unlikely, but not impossible, that the LPS of these bacteria would have been bound by the anti-cLPS antibody. Finally, since the antibody used in the EIA method is *Chlamydia* genus-specific, the cLPS of *Chlamydia* species other than *C. pneumoniae* may also have been detected, even though their amount in atherosclerotic lesions is probably low. Overall, detection of cLPS from homogenized atherosclerotic tissue with the commercial EIA kit was rapid and easy, and unlike a PCR, the EIA method should not be especially prone to inhibition and contamination.

**Serum samples (IV)**

Serum cLPS was measured with a recently developed, novel EIA method in which serum LPS is first captured by LBP, LBP-cLPS complexes are then bound to a solid phase by a monoclonal anti-cLPS antibody, and then detected with an anti-LBP antibody (Tiirola *et al.* 2006). The advantage of this method is that it measures the concentration of cLPS in the samples and is not dependent on the activity of LPS.

Serum totLPS was determined with a kinetic Limulus Amebocyte Lysate (LAL) test kit, the principle of which is based on the fact that bacteria cause intravascular coagulation in the American horseshoe crab (*Limulus polyphemus*). In this assay LPS causes an enzymatic reaction that leads to opacity and gelation in LAL, and the assay thus measures the activity of LPS in the samples.
6.2.6 Serum levels of C. pneumoniae antibodies (IV)

Serum C. pneumoniae antibodies were measured by the MIF method, which is known to be a sensitive and specific method when performed properly (Peeling et al. 2000, Dowell et al. 2001). Experienced laboratory technicians performed the assay, and all the results were read by one experienced reader. Gullsorb treatment was used for measurement of IgA antibodies as has been recommended to prevent interference by IgG antibodies (Jauhiainen et al. 1994). Unfortunately, we did not have a possibility to measure the persistence of the antibodies, since there were no follow-up serum samples available, thus it is not possible to say if the elevated antibody levels were due to past or persistent infections. Acute infections as a cause of elevated antibody levels are not likely in our study populations, since patients with acute symptomatic respiratory infections are not normally operated on, and also due to regulations concerning blood donation (see chapter 6.1 Study populations).

6.2.7 Serum levels of cholesterol, triglycerides, and inflammatory and innate immunity markers (II, IV)

The serum levels of total cholesterol, HDL, and triglycerides were measured with photometric enzymatic methods as routine measurements in the Helsinki University Central Hospital laboratory (IV). LDL levels, however, were calculated with the Friedewald formula: TC = (VLDL-C + HDL-C), and VLDL-C = TG/2.2, in which TC is total cholesterol, VLDL-C is very-low-density lipoprotein cholesterol, HDL-C is high-density lipoprotein cholesterol, TG is triglycerides, and 2.2 is a constant when the measurements are in mmol/l. Use of this formula is not recommended if TG > 4.52 mmol/l or if chylomicrons are present in the samples. This formula is commonly used because direct measurement of LDL-C is rather difficult and causes extra costs. However, several limitations that may cause inaccuracy in the results have been reported. Calculation of LDL may not be accurate due to 1. cumulative error from measurements of TC, HDL-C, and TG; 2. the fact that the ratio of VLDL-C to TG changes gradually when the TG level increases; and 3. inaccuracy at low serum levels of LDL-C. (Sniderman et al. 2003.)

The serum levels of IL-6, hsCRP, LBP, and sCD14 were measured with commercially available kits, according to the manufacturers’ instructions (II, IV). Some inaccuracy may have been introduced, since only one measurement per
sample was done. However, the negative and positive controls performed well in all the measurements and some samples from previous series were included in each run in order to monitor the overall level of the measurements.

6.3 The main findings

6.3.1 Gene polymorphisms (II)

At least weak positive correlations were observed in the blood donor group between all the serum concentrations of the inflammatory and innate immunity markers, and many of these associations were also seen in the multivariate analyses. Similar associations have been reported by others, and they seem logical, since IL-6 has been reported to regulate expression of CRP, LBP, and sCD14 (Grube et al. 1994, Wan et al. 1995, Schumann et al. 1996, Bas et al. 2004).

LBP and TLR-4 polymorphisms

The $LBP_{C1341\rightarrow T}$ (Leu436→Phe) and $TLR-4_{A896\rightarrow G}$ (Asp299→Gly) polymorphisms were found to be associated with serum LBP concentration. The C allele of the $LBP$ polymorphism was associated with increased serum LBP, especially among the men. The $TLR-4$ polymorphism allele G was associated with decreased serum LBP among the women, but there was a trend of the $TLR-4$ GG homozygotes having lower serum LBP levels among the men (data not shown). These effects of the $LBP$ and $TLR-4$ polymorphisms on LBP concentration were found to be independent of each other. The mechanism behind the gender dependence of these associations is not clear, but gender has been suggested to have an effect on the intensity of innate immunity responses (Imahara et al. 2005, Marriott et al. 2006). In addition, similar situations where a certain polymorphism seems to have an effect on certain traits among only either men or women have been reported by other studies (Eklund et al. 2008), and this has also been demonstrated for the $TLR-4$ polymorphism (Ådjers et al. 2005).

In the case of the $LBP$ polymorphism, the highest mean serum LBP concentrations among the whole study population were found in heterozygous individuals, but among the men in CC homozygotes. However, there were only four CC homozygotes in this study and, thus, the mean value for this group alone is not descriptive. The mechanism behind the association between the C allele and
higher serum LBP observed in our study is not clear. One possibility is that since the polymorphism lies in the C-terminal region of the LBP protein that contains the domain responsible for interaction with CD14 (Theofan et al. 1994), the polymorphism may affect the function of LBP. Perhaps interaction of the molecules and the subsequent cell activation are more efficient with the C allele than with the T allele, which would again result in higher LBP expression. It is also possible that LBP concentration is affected by other polymorphisms that were not studied here. For example, Chien et al. (2008) reported an association between LPB concentration and a polymorphism (SNP 1683, rs2232571) in the promoter region of the LBP gene. They found that the promoter variant associated with elevated serum LBP was also associated with a fivefold higher risk of death due to Gram-negative bacteremia after transplantation (Chien et al. 2008). In light of this finding, one might speculate that the slightly increased, though not statistically significant, frequency of CC homozygotes in the sepsis non-survivors group in the study by Hubaceck et al. (2001) could be due to an elevated LBP concentration. It is also possible that these two polymorphisms (rs2232618 and rs2232571) together regulate serum LBP concentration, and it would be interesting to study their combined effect.

Our finding of the association between the TLR-4 polymorphism and serum LBP is in concordance with previous publications that suggest that the TLR-4 G (Gly) allele is LPS-hyporesponsive (Arbour et al. 2000, Schmitt et al. 2002). This nonsynonymous polymorphism is located near the MD-2 binding area of TLR-4, and may cause increased rotation of the peptide bond and remove the negative charge of the area, thus possibly affecting the functionality of the receptor (Rallabhandi et al. 2006). Perhaps hyporesponsiveness also results in reduced LBP expression following cell activation (Grube et al. 1994, Wan et al. 1995). At least one previous study has reported findings similar to ours; after LPS inhalation, persons heterozygous for the TLR-4 A896→G polymorphism had lower serum levels of LBP and CRP and a lower white blood cell count than AA homozygotes. However, they found no gender dependence and there were no G allele homozygotes in their study. (Michel et al. 2003.) The functionality of the polymorphism is supported by studies reporting that the G allele may also associate with a higher risk of infections (Agnese et al. 2002, Tal et al. 2004, Montes et al. 2006), sepsis (Barber et al. 2006), malaria (Mockenhaupt et al. 2006), periodontitis (Schröder et al. 2005), and cardiovascular diseases (Vainas et al. 2006). However, there are also many studies that did not find any association (Ahrens et al. 2004, Morange et al. 2004, Laine et al. 2005, Lalouschek et al. 2006).
In European populations the 896G (299Gly) allele has been shown to cosegregate with the 399Ile allele of the TLR-4 (Thr399→Ile, rs4986791) polymorphism (Arbour et al. 2000, Ferwerda et al. 2007). This cosegregation has also been demonstrated in a Finnish population, where 98.6% of cases carrying the 299Gly allele also had the 399Ile allele (Lorenz et al. 2002a). It has recently been suggested that the 299Gly allele alone, but not in combination with 399Ile allele, would actually be associated with a proinflammatory phenotype, and that the combination of 299Gly + 399Ile would not functionally differ from a wild type receptor (Ferwerda et al. 2007). Similarly, the 299Gly allele alone has been suggested to associate with septic shock, whereas no association has been seen for the 299Gly + 399Ile combination (Lorenz et al. 2002b). Here, we did not genotype the Thr399→Ile polymorphism, but it would be interesting to study what proportion of 896G (299Gly) carriers would also carry the 399Ile allele, and whether this would modify the association with serum LBP levels.

**CD14 polymorphism**

There was a trend towards the T allele of the CD14 C–260→T polymorphism being associated with increased serum sCD14 among the men. However, this association did not reach statistical significance after correction for multiple testing. Our finding is in accordance with previous studies that have reported an association between either the TT genotype or T allele carriage and a higher sCD14 concentration (Baldini et al. 1999, Karhukorpi et al. 2002, Eng et al. 2004) or mCD14 density (Hubacek et al. 1999, Eng et al. 2004). However, contrasting studies that found no association between the genotypes and sCD14 and/or mCD14 also exist (Heesen et al. 2001). The actual difference in sCD14 concentration between the genotypes in our study was small, but similar to those previously reported in some of the studies (Karhukorpi et al. 2002, Eng et al. 2004). In addition, Amar et al. (2004) reported that in current smokers, there was no difference in sCD14 concentration between the genotypes, whereas among non-smokers, the T allele was associated with higher sCD14 levels. Since we could not analyze the effect of smoking, it is possible that the presence of smokers in our data attenuated the difference between the genotypes. Our study implicates that the T allele may increase sCD14 expression.
**IL-6 polymorphism**

The *IL-6* G_{-174}→C polymorphism genotype GG and the G allele were associated with a higher prevalence of *C. pneumoniae* antibodies. In a multivariate analysis, the G allele increased the risk of having *C. pneumoniae* IgG ≥ 64 and/or IgA ≥ 20 by 1.8-fold. Some previous studies have shown that the G allele would be associated with increased IL-6 expression and serum levels (Fishman et al. 1998, Hulkkonen et al. 2001, Rivera-Chavez et al. 2003). However, also opposite findings (Jones et al. 2001, Kilpinen et al. 2001) and studies that showed no association exist (Hulkkonen et al. 2000, Veres et al. 2002, Eklund et al. 2006, Sie et al. 2006, Hegedus et al. 2007). No association between the *IL-6* polymorphism and serum IL-6 concentration was found in our study. Serum IL-6 concentration is, however, affected by many factors that were not measured in our study, such as BMI, smoking, time of day when a blood sample was taken (Gudewill et al. 1992), and stress (Steptoe et al. 2007), which may have confounded our analysis of IL-6 concentration. Our results suggest that the *IL-6* polymorphism may increase susceptibility to *C. pneumoniae* infection, but the possible mechanism is unclear. The *C. pneumoniae* antibody titre limits used here were relatively high (IgG ≥ 64 and/or IgA ≥ 20), which may indicate either recent or persistent/chronic *C. pneumoniae* infection. Similar results have also been reported for the association of the C allele with remarkably lower serum levels of anti-hHsp60 and anti-*Mycobacterium bovis* Hsp65 antibodies (Veres et al. 2002, Pandey et al. 2004, Kiszel et al. 2006, Hegedus et al. 2007). Interestingly, the disease associations reported for the *IL-6* polymorphism include e.g. systemic-onset juvenile chronic arthritis (Fishman et al. 1998), Alzheimer’s disease (Shibata et al. 2002), and peripheral artery occlusive disease (Flex et al. 2002), most of which have also been associated with *C. pneumoniae* infection (Taylor-Robinson et al. 1998, Linares-Palomino et al. 2004, Gérard et al. 2006).

**TLR-2 polymorphism**

No association of the *TLR2* G_{2408}→A (Arg_{753}→Gln) polymorphism with any of the markers of inflammation, innate immunity, or *C. pneumoniae* infection markers was seen in this study, possibly due to confounding factors that could not be taken into account and the very low number of A allele carriers in our study population.
6.3.2 Use of the cLPS EIA method in the detection of C. pneumoniae with atherosclerotic tissue samples and a comparison of the PCR, ISH, and EIA methods (III)

Different methods of detecting *C. pneumoniae* in atherosclerotic tissue of patients with CAR were compared. To our knowledge, only one previous study has used the EIA method to measure the presence of chlamydial LPS in atherosclerotic tissue. In that study, the cLPS positivity rate in lesions of patients with aortoiliac occlusive disease was 19.05% (Kaklikkaya *et al.* 2006), which is similar to that observed in our study (22.2%). By IEM, we could also demonstrate that cLPS associated with cell membranes, suggesting that the presence of cLPS may not always indicate the presence of the organism itself at that specific site of a lesion. This is in line with previous studies that have reported finding only chlamydial components and not live bacteria in atherosclerotic vascular tissue or abdominal aortic aneurysms (Meijer *et al.* 1999, Meijer *et al.* 2000). However, the presence of cLPS itself may also contribute to the pathogenesis of atherosclerosis through the induction of macrophage activation and foam cell formation (Kalayoglu & Byrne 1998a, Kalayoglu & Byrne 1998b, Kalayoglu *et al.* 1999).

When the results of PCR, ISH, and cLPS EIA were compared, a poor concordance between the different methods was observed. The highest positivity rate was seen in the ISH method (39.4%), and the lowest in the cLPS EIA method (22.2%). Of the tissue samples of the patients tested with all of the methods, 65.3% were positive by at least one method, but only 9.3% of them were positive by all of the methods. This may be explained, at least partly, by the poor repeatability of the PCR methods and the technical problems inherent in ISH and, thus, by the possible false negative and positive results yielded by them.

6.3.3 Association of cLPS with elevated BMI in patients with CVD (IV)

*C. pneumoniae* IgG, but not IgA, antibody positivity and higher serum cLPS concentration were found to associate with an elevated BMI. Several previous studies have also reported an association between *C. pneumoniae* IgG positivity and an elevated BMI in different study populations (Ekesbo *et al.* 2000, Dart *et al.* 2002, Karppinen *et al.* 2003, Saikku *et al.* 2004, Thjodleifsson *et al.* 2007). However, there are also some studies that did not find an association (Kaftan & Kaftan 2000, Koziolek *et al.* 2008). To our knowledge, our study is the first to report an association between serum cLPS levels and BMI. This finding further
strengthens the theory that there may be a link between chlamydial infection and obesity, since the presence of cLPS in serum probably requires active productive infection. In the case of our study, such active infections are likely to be subclinical and chronic in nature, since patients with clinical acute infection are not normally operated on. However, since the antibody used in the cLPS EIA is Chlamydia genus-specific, some of the detected cLPS may have originated from chlamydial infections other than C. pneumoniae. On the other hand, C. trachomatis infections are not common in the elderly population, and their effect on cLPS levels in serum can be considered negligible. Our study also suggests that the association between infection and an elevated BMI may be specific to certain pathogens and not to e.g. Gram-negative bacteria in general, since no association was found between serum total endotoxin activity and BMI. On the contrary, however, obesity and metabolic endotoxemia have been found to be linked in animal experiments (Cani et al. 2007, Cani et al. 2008). Interestingly, no correlation was observed between serum cLPS concentration and total endotoxin activity. This most probably is due to differences in the methods used to detect cLPS and totLPS. In addition, serum cLPS only forms a small portion of the total LPS concentration in circulation.

Even though an association between infections and obesity has been demonstrated in several studies, the causality between these two is not known. It has been shown in mice and humans that obesity may be associated with impaired immune responses (Tanaka et al. 1993, Amar et al. 2007), and overweight has been suggested to be a risk factor of respiratory infections (Jedrychowski et al. 1998). In the case of chlamydial infections, an impaired immune response might predispose, not only to acute infections, but also to persistence of infection. On the other hand, several infectious agents like avian adenovirus SMAM-1 and Adenovirus-36 have been reported to possibly promote obesity (Dhurandhar et al. 1997, Dhurandhar 2001, Atkinson et al. 2005, So et al. 2005, Pasarica et al. 2006). In addition, Ad-36 can infect adipocytes and induce adipogenesis in primary human adipose-derived stem cells (Rogers et al. 2007). Interestingly, two recent studies have shown that C. pneumoniae is also capable of infecting murine and human adipose tissue cells (Bouwman et al. 2008, Shi et al. 2008). Furthermore, in C. pneumoniae-infected LDLR−/− mice, the pathogen was found in preadipocytes in the adipose tissue (Shi et al. 2008). In this light, our finding that the serum level of cLPS increased with increasing BMI may suggest that at least part of cLPS could have been released from infected adipose tissue.
In an obese state, macrophage accumulation and activation is seen in adipose tissue (Cancello & Clément 2006). Since *C. pneumoniae* is capable of infecting and surviving in monocytes/macrophages, it may through them have “access” to adipose tissue, where it may then further infect adipocytes. Adipose tissue is known to express inflammatory factors, which are mainly produced by the macrophages of the stromal vascular fraction, but also by adipocytes (Cancello & Clément 2006, Desruisseaux *et al.* 2007). *C. pneumoniae* infection in adipose tissue could also promote production of inflammatory molecules from both macrophages and adipocytes (Gaydos *et al.* 1996, Kaukoranta-Tolvanen *et al.* 1996b, Shi *et al.* 2008), and even accumulation of fat inside both cell types (Kalayoglu & Byrne 1998b, Hyvärinen *et al.* 2007).

In addition to local inflammation in adipose tissue, obesity is associated with elevated circulating levels of inflammatory molecules like IL-6 and CRP (Bastard *et al.* 2006). An association between BMI and hsCRP was observed also in our study. In addition, higher serum cLPS, *C. pneumoniae* IgG positivity, and smoking were associated with higher serum hsCRP. Besides from adipose tissue, serum cLPS could have originated e.g. from infection in the lungs, aneurysms, or atherosclerotic plaques. Thus, chlamydial infection might also generally promote inflammation and increase the levels of serum inflammatory markers, which again may contribute to development of obesity and inflammation-related conditions like decreased insulin sensitivity, type 2 diabetes, metabolic syndrome, and CVD (Pickup & Crook 1998, Ross 1999, Festa *et al.* 2000, Yudkin *et al.* 2000, Ritchie & Connell 2007).
7 Conclusions

In this work we established a new, reliable and fast method for genotyping the $\text{LBP}_{1341\to T}$ (Leu$^{436\to Phe}$, rs2232618) polymorphism. Serum levels of inflammatory and innate immunity markers, namely IL-6, CRP, LBP, and sCD14, in apparently healthy individuals were found to correlate with each other and possibly be regulated by the polymorphisms of genes important in innate immunity. Especially serum LBP levels may be regulated by the $\text{LBP}_{1341\to T}$ (Leu$^{436\to Phe}$, rs2232618) and $\text{TLR-4}_{A896\to G}$ (Asp$^{299\to Gly}$, rs4986790) polymorphisms (Fig. 6). The only gene polymorphism associated with markers of $C.\ pneumoniae$ infection was $\text{IL-6}_{G^{174\to C}}$, the $G$ allele of which was associated with a higher risk for $C.\ pneumoniae$ antibody positivity (Fig. 6). Further studies with larger study populations are needed to clarify the association of the $\text{LBP}_{1341\to T}$ (Leu$^{436\to Phe}$) polymorphism with serum LBP levels, and if this is confirmed, the possible mechanism should then be studied. It would also be interesting to study the interaction between $\text{LBP}$ polymorphisms (rs2232618 and rs2232571) on serum LBP levels. Also the association of the $\text{IL-6}_{G^{174\to C}}$ polymorphism with $C.\ pneumoniae$ antibody levels should be confirmed in another population.

$C.\ pneumoniae$ DNA and cLPS can be found from atherosclerotic tissue (Fig. 6). The chlamydial LPS EIA method was easy to use and its advantage is that it should not be particularly susceptible to contamination or inhibition, like PCR methods are. If the sensitivity of the EIA method could be increased, for example, by testing multiple pieces of tissue, cLPS EIA might provide a standardized, commercial method for detecting chlamydia in tissue samples, and thus it merits further characterization and validation in different patient populations.

$C.\ pneumoniae$ IgG positivity and elevated serum cLPS levels were associated with an elevated BMI. Chlamydial LPS released from e.g. infected adipose tissue may lead to increased production of inflammatory molecules deriving from both macrophages and adipocytes in adipose tissue, thereby further explaining the observed associations between serum hsCRP, cLPS, and BMI. Serum cLPS could also originate from infection in sites other than adipose tissue. In this way, chlamydial infection might also promote generalized inflammation in obese individuals. (Fig. 6.) The lack of an association between totLPS and BMI implies that the association between infection and an elevated BMI may be specific to certain pathogens. In future studies, the association of cLPS with BMI...
should be confirmed in a normal population, and the effect of *C. pneumoniae* infection in adipocytes should be further clarified.

Fig. 6. The main findings of this work and their possible role in pathogenesis of atherosclerosis.
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