Aino Rantala

SUSCEPTIBILITY TO RESPIRATORY TRACT INFECTIONS IN YOUNG MEN: THE ROLE OF INFLAMMATION, MANNOSE-BINDING LECTIN, INTERLEUKIN-6 AND THEIR GENETIC POLYMORPHISMS

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Abstract

Respiratory tract infections are the most common acute illnesses, and innate immunity and inflammation are important in defence against these infections. Mannose-binding lectin (MBL) mediates innate immune defences by recognising microbial structures. MBL deficiency caused by polymorphisms in the MBL2 gene has been associated with susceptibility to recurrent infections. Interleukin-6 (IL-6) is a mediator of inflammatory response. Polymorphisms in the IL-6 and IL-6 receptor (IL-6R) genes have been previously associated mainly with metabolic disorders and cardiovascular diseases. Chlamydia pneumoniae is a common pathogen in acute respiratory tract infections, but it also has a tendency to cause persistent infections, which have been associated with cardiovascular diseases and its risk factors, such as obesity.

The aims of this study were to investigate if selected polymorphisms of the MBL2, IL-6 and IL-6R genes are associated with respiratory tract infections and markers of C. pneumoniae infection, and to study if persistent C. pneumoniae infection is connected with an elevated body mass index (BMI) in 893 Finnish male military conscripts. Respiratory tract infections were followed during their military service and serum samples were collected at the beginning and end of their service and during each infectious episode.

A variation in serum MBL levels between different MBL2 genotypes and a MBL deficiency in homozygous exon 1 variant genotypes (at codons 52, 54 and 57) were observed. Low MBL levels and MBL2 polymorphisms in exon 1 and promoter region were found to be risk factors for susceptibility to respiratory tract infections as well as for positivity and a rise in C. pneumoniae antibodies during military service.

Associations between IL-6R gene polymorphisms in the promoter region (-183G/A) and in intron 1 and respiratory tract infections were found. In addition, the IL-6 -174G/C polymorphism was associated with persistently elevated C. pneumoniae antibodies and with slightly elevated serum C-reactive protein (CRP) levels, pointing to chronic C. pneumoniae infection.

Furthermore, persistent C. pneumoniae antibodies as a suggestive marker of chronic infection, and elevated serum CRP levels as a marker of systemic inflammation, were associated with an elevated BMI.

In conclusion, the findings support the role for MBL in susceptibility to infections and provide new information about the association between MBL and common respiratory tract infections. The results also suggest that the 5' area of the IL-6R gene may be a possible candidate region for respiratory tract infection susceptibility, and that IL-6 genetics may be associated with C. pneumoniae infection. The study also provides new information about the role of possible chronic C. pneumoniae infection in obesity.

Keywords: body mass index, Chlamydia pneumoniae, genetic polymorphism, inflammation, innate immunity, interleukin-6, mannose-binding lectin, military conscript, respiratory tract infections
Rantala, Aino, Inflammaatio, mannoosia sitova lektiini, interleukiini-6 ja niiden geenipolymorfismit: vaikutus nuorten miesten hengitystieinfektiointiin

Lääketieteellinen tiedekunta, Diagnostiikan laitos, Lääketieteellinen mikrobiologia, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto; Turveyden ja hyvinvoinnin laitos, Lasten ja nuorten hyvinvointi ja terveys, PL 310, 90101 Oulu; Puolustusvoimat, Sotilaslääketieteen keskus, PL 2, 15701 Lahti

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Tiivistelmä

Hengitystieinfektiot ovat yleisimpiä äkillisiä sairauksia, ja synnynnäisellä immuunivasteella ja tulehduksella on tärkeä rooli puolustuksessa näitä infektioita vastaan. Synnynnäiseen immunitteettiin kuuluva mannoosia sitova lektiini (MBL) tunnistaa infektioita aiheuttavien mikrobien rakenteita. MBL2-geenin polymorfismien aiheuttaman MBL-proteiinin puuteen on todettu altistavan toistuville infektioille. Interleukiini-6 (IL-6) on tulehduksen välittäjänä toimiva sytokiini. IL-6- ja IL-6-reseptori (IL-6R) -geenien polymorfismit on aiakaisemmin yhdistetty lähinnä metabolisiin häiriöihin sekä sydän- ja verisuonitauteihin.

Chlamydia pneumoniae eli keuhkoklamidia on yleinen hengitystieinfektioiden aiheuttaja, mutta se voi myös aiheuttaa kronisia infektioita, jotka on yhdistetty sydän- ja verisuonitauteihin sekä niiden riskitekijöihin kuten lihavuuteen.

Työn tarkoituksena oli tutkia tiettyjen MBL2-, IL-6- ja IL-6R-geenien polymorfismit yhteyttä hengitystieinfektiioihin ja keuhkoklamidyaviasta-ainetaoihin sekä keuhkoklamidyainfektion yhteyttä painoindeksiin 893 suomalaisella varusmiehellä. Hengitystieinfektiota seurattiin palveluksen aikana, ja seeruminäytteet kerättiin palveluksen alussa, lopussa ja jokaisen infektion aikana.

Tutkimuksessa havaittiin vaihtelua seerumin MBL-pitoisuudessa eri MBL2-genotyyppien välillä sekä MBL:n puute homotsygooteissa eksoni 1 -alueen variantit (kodonesi 52, 54 ja 57). Alhaiset MBL-tasot sekä MBL2-geenin polymorfismit eksoni 1 -alueella ja säätelyalueella olivat riskitekijöitä hengitystieinfektiointiin sekä keuhkoklamidyaviastaa-ainetaoihin, mikäli esintymiseen ja vasta-ainetedien nousulle palveluksen aikana.

IL-6R-geenin polymorfismit säätelyalueella (-183G/A) ja introni 1 -alueella liittyivät hengitystieinfektiioihin. Lisäksi IL-6-geenin -174G/C polymorfismi oli yhteydessä jatkuvasti kohonneeniin keuhkoklamidyaviasta-ainetaoihin sekä seerumin C-reactiivisen proteiinin (CRP) tasoihin, jotka mahdollisesti osoittaisivat kronista keuhkoklamidyainfektioita. Lisäksi kroniseen keuhkoklamida-infektioon viittaavat vasta-ainetasot sekä tulehduksen aikana liittyvä nopeutunut kohonnut CRP-positiiviset olivat yhteydessä ylipainoon.

Tutkimuksen tulokset tukevat aiakaisemmin havaittua MBL:n vaikutusta infektioalttiuteen ja lisäksi antavat uutta tietoa MBL:n yhteydestä tavallisiihin hengitystieinfektiioihin. Tulokset viittavat myös siihen, että IL-6-geenin 5'-alueella voi olla yhteyttä hengitystieinfektiointiin ja että IL-6-polymorfismi olisi yhteydessä keuhkoklamidyainfektioon. Tutkimus antaa myös uutta tietoa mahdollisen kroonisen keuhkoklamidyainfektion liittymisestä ylipainoon.

Asiasanat: Chlamydia pneumoniae, geenipolymorfismi, hengitystieinfektiio, interleukiini-6, mannoosia sitova lektiini, painoindeksi, synnynnäinen immunitetti, tulehduas, varusmiehet
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Abbreviations

AIDS acquired immune deficiency syndrome
BMI body mass index
CD14 cluster of differentiation 14
CHD coronary heart disease
CI confidence interval
CRD carboxyterminal recognition domain
CRP C-reactive protein
DNA deoxyribonucleic acid
EB elementary body
ELISA enzyme-linked immunosorbent assay
HDL high-density lipoprotein
HIV human immunodeficiency virus
hsCRP high-sensitivity C-reactive protein
Hsp heat shock protein
IEMA immunoenzymometric assay
IFN interferon
Ig immunoglobulin
IL interleukin
IL-6R interleukin-6 receptor
IRF interferon regulatory factor
JAK Janus kinase
LBP lipopolysaccharide binding protein
LD linkage disequilibrium
LDL low-density lipoprotein
LPS lipopolysaccharide
MAC membrane-attack complex
MAF minor allele frequency
MASP MBL-associated serine protease
MBL mannose-binding lectin
MD median
MDA melanoma differentiation-associated
MIF microimmunofluorescence
mRNA messenger ribonucleic acid
NF-κB nuclear factor-κB
NOD nucleotide-binding and oligomerisation domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RAV-7</td>
<td>rous-associated virus-7</td>
</tr>
<tr>
<td>RB</td>
<td>reticulate body</td>
</tr>
<tr>
<td>RIG</td>
<td>retinoic acid-inducible gene</td>
</tr>
<tr>
<td>RLR</td>
<td>retinoic acid-inducible gene (RIG)-I-like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>surfactant protein</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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1 Introduction

Respiratory tract infections are the most common acute illnesses. Typical risk factors for respiratory infections are young age and crowded living conditions, e.g. day-care centres and military service. Innate immunity acts as a first-line defence against infectious agents. Genetic variations in molecules that act in innate immune responses predispose to susceptibility to infections.

Mannose-binding lectin (MBL) is a serum collectin and also present in the respiratory airways. MBL mediates the innate defences by recognising the carbohydrate structures of microbes and then acting as an opsonin for phagocytosis and activating the complement lectin pathway. Three single-nucleotide polymorphisms (SNPs) in the exon 1 region and three in the promoter region of the \( MBL2 \) gene have an effect on serum MBL concentration. MBL deficiency caused by the SNPs has been associated with susceptibility to recurrent infections in several studies. MBL deficiency is often considered a risk factor only for those who also have some other defect in immunity (Garred et al. 2006, Turner 2003). Several studies have shown that MBL deficiency is a risk factor for respiratory tract infections in children (Chen et al. 2009, Koch et al. 2001, Summerfield et al. 1997), but only a few studies have been reported in adults (Gomi et al. 2004, Ruskamp et al. 2006). Earlier findings have indicated that MBL may play a role in protecting against \( Chlamydia pneumoniae \), a common pathogen in acute respiratory tract infections in humans, by inhibiting infection of the host cells in vitro (Swanson et al. 1998). However, an association between MBL and seropositivity for \( C. pneumoniae \) has not been reported.

Interleukin-6 (IL-6) is a mediator of inflammatory responses in the host’s innate defence against infection. The action of IL-6 is mediated by an IL-6 receptor (IL-6R). High levels of IL-6 and IL-6R and SNPs in these genes have been previously associated mainly with metabolic disorders and cardiovascular diseases (Bustamante et al. 2007, Hamid et al. 2004, Humphries et al. 2001, Ridker et al. 2000b, Vozarova et al. 2003). IL-6 -174 G/C SNP has also been associated with a risk of upper respiratory tract infections and susceptibility to otitis media in children (Patel et al. 2006, Revai et al. 2009), as well as with longevity (Hurme et al. 2005). However, the association between IL-6R and respiratory tract infections has not been studied.

\( C. pneumoniae \) infection has a tendency to cause persistent infections, which have been associated with chronic diseases such as asthma and cardiovascular diseases (Saikku 2002). Recent studies have reported an association between
elevated *C. pneumoniae* antibodies and obesity (Karppinen et al. 2003, Lajunen et al. 2008a). Obesity is considered an inflammatory disease, where the expression of proinflammatory cytokines, including IL-6, is increased. IL-6 is a stimulator of C-reactive protein (CRP), which is a marker of low-grade inflammation. Low-grade inflammation induces insulin resistance and obesity, which are linked to metabolic disorders and cardiovascular diseases. (Cancello & Clement 2006.) The association between seropositivity for *C. pneumoniae* and IL-6 has not been reported before, but *C. pneumoniae* infection has been shown to induce production of IL-6 in macrophages (Kaukoranta-Tolvanen et al. 1996).

This study included a healthy, young population of Finnish military recruits. The questionnaire data were collected at the beginning of their military service, respiratory tract infections were followed during their service and serum samples were collected at the beginning and end of their service and during each infectious episode. The material enabled a study of recurrent respiratory infections as well as *C. pneumoniae* exposure, persistence of *C. pneumoniae* antibodies and low-grade inflammation during that time. In addition, a relatively high number of infectious episodes provided a good opportunity to investigate genetic susceptibility to infections.
2 Review of literature

2.1 Respiratory tract infections

Respiratory tract infections are divided into upper and lower respiratory tract infections. Acute upper respiratory tract infections are frequent and often minor and self-limiting illnesses, such as a common cold, otitis media, sinusitis, pharyngitis and tonsillitis. They are the most common acute illnesses and a significant cause of school and work absence and increased visits to doctors worldwide. Viruses have the most crucial role in the aetiology of upper respiratory tract infections. A large number of viruses can cause infections, but rhinoviruses are the most frequent causative agent, especially in mild infections or common colds (Mäkelä et al. 1998). Other causative viruses include e.g. coronavirus, influenza A and B virus, parainfluenza virus, respiratory syncytial virus (RSV), adenovirus, enterovirus and the newly identified metapneumovirus and human bocavirus. Rhinoviruses are found in 30–50%, coronaviruses in 7–18% and other viruses in a minor proportion of adults with upper respiratory tract infections (Heikkinen & Järvinen 2003, Mäkelä et al. 1998, Wat 2004). In addition, double viral infections have been reported in 5% of patients with a common cold (Drews et al. 1997, Mäkelä et al. 1998). Bacterial agents are also common in the aetiology. Especially, beta-hemolytic group A Streptococcus and Chlamydia pneumoniae are seen in pharyngitis and Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis in acute otitis media. Concomitant bacterial infections are also recognised in viral infections. Bacteria may asymptomatically colonise the nasopharynx, but in some patients secondary bacterial infections develop during or after viral infection. (Heikkinen & Järvinen 2003, Ruuskanen & Heikkinen 2003, Wat 2004.)

Lower respiratory tract infections, such as pneumonia and bronchitis, are more severe illnesses that can cause death, especially in children, immunocompromised patients and the elderly. *S. pneumoniae* is the most prevalent aetiological agent in pneumonia, causing about 20–50% of pneumonia cases. Other pathogens in pneumonia include *H. influenzae, M. catarrhalis, C. pneumoniae* and *Mycoplasma pneumoniae* as well as viruses, like influenza and RSV. Mixed infections are also recognised. Acute bronchitis is caused mainly by viruses, e.g. rhinovirus, coronavirus, influenza and adenovirus, but also by
bacteria, like *M. pneumoniae* and *C. pneumoniae*. (Guthrie 2001, Korppi et al. 2003.)

Respiratory tract infections occur with seasonal variation. For example, rhinoviruses can cause infections all year round, but a peak of infections is seen in autumn, whereas influenza epidemics typically occur in winter. The number of respiratory infections decreases with age; children up to 4 years of age are most susceptible to infections. Crowded living conditions in day-care centres and military service increase the risk of infection transmission. Respiratory infections have been reported to be more common in women who stay at home than in those who work outside the home, possibly due to their greater exposure to children in the family. (Heikkinen & Järvinen 2003, Monto 2002.)

### 2.1.1 Innate immunity to respiratory tract infections

The innate immune system is a non-specific immune response that protects the host as a first-line defence against infectious agents. It is essential in quickly recognising and responding to an infective agent before the specific adaptive immune system becomes operative. Innate immunity is especially important at an early age, between 6 and 18 months when the adaptive system is still immature, and in immunocompromised persons.

Failure of the local defence system in the respiratory tract may cause microbial colonisation and lead to an infection of the airways. The respiratory tract’s epithelium represents the primary site for the innate host defence against invading pathogens. It acts as a physical and mechanical barrier against the pathogens: the airway epithelium is covered by mucus and the cilia on the epithelium clear away microorganisms. Airway epithelial cells secrete several antimicrobial molecules, including α- and β-defensins and cathelicidins, which are important in the innate defence and inflammatory reactions against microorganisms until they are eliminated by recruited phagocytes and the adaptive immune system has developed. In addition, a wide range of innate immune components that function as pattern recognition molecules are located in the respiratory airways. These molecules recognise specific microbial structures, including lipids, lipoproteins, proteins, carbohydrates and nucleic acids. The response to microbial ligands mediates the transcription of a variety of genes involved in inflammatory responses. The inflammatory mechanisms mediated by pattern recognition molecules are important as they increase the release of cytokines that then activate phagocytic cells and antigen-presenting cells and

**Pattern recognition molecules**

**Toll-like receptors.** Pattern recognition molecules include a large family of Toll-like receptors (TLR). Ten TLRs that have been recognised in humans are described in Table 1. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell surface of epithelial cells and in immune cells, including macrophages, dendritic cells, B cells and T cells, where they recognise microbial molecules. TLR4 recognises lipopolysaccharide (LPS), the outer membrane component of Gram-negative bacteria. This recognition is mediated by a lipopolysaccharide-binding protein (LBP), an acute-phase protein present in the serum that binds to bacterial LPS. LBP transfers the LPS to cluster of differentiation 14 (CD14), other pattern recognition receptor proteins and a part of the TLR4 receptor complex. Then the LPS is transferred to MD-2, which associates with TLR4. Another important TLR in the host’s defence against bacterial pathogens of the respiratory airways is TLR2, which is activated by the cell surface components: peptidoglycan and lipopeptides. During chlamydial infection, TLR2 acts as a receptor for lipopeptides and TLR4 for LPS. (Bals & Hiemstra 2004, Kawai & Akira 2009, Kumar et al. 2009, Takeuchi & Akira 2010.)

The intracellular TLRs, including TLR3, TLR7, TLR8 and TLR9, are expressed in the endosomal membrane and they recognise internalised nucleic acids. TLR9 mediates the response to bacterial deoxyribonucleic acid (DNA) and TLR3 is an important receptor for double-stranded ribonucleic acid (RNA) produced during viral infections. TLR3 has been shown to be important in rhinovirus infections. (Kawai & Akira 2009, Takeuchi & Akira 2010.)

Recognition of ligands by TLR leads to upregulation of a variety of genes that encode inflammatory cytokines, chemokines and antimicrobial peptides. Ligand binding activates the intracellular signalling pathway where nuclear factor (NF)-κB and interferon regulatory factor (IRF) mediate the transcription of inflammatory genes. (Takeuchi & Akira 2010.)

**Non-toll-like receptors.** Non-toll-like receptors include cytosolic nucleotide-binding and oligomerisation domain (NOD) proteins, retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and C-type lectin receptors (Table 1). NOD receptors recognise intracellular bacteria (Sabbah et al. 2009). NOD1 is involved in the immune response against e.g. *H. influenzae* and *C. pneumoniae*. NOD2
participates in the response against *Mycobacterium tuberculosis* and *S. pneumoniae*. (Carneiro *et al.* 2007, Kawai & Akira 2009.) RLR molecules such as RIG-I and melanoma differentiation-associated gene 5 (MDA5) are localised in the cytoplasm and recognise RNA viruses. C-type lectins such as mannose receptors, dectin-1 and dectin-2 are transmembrane receptors that recognise carbohydrate structures on viruses, bacteria and fungi. The activation of non-toll-like receptors results in NF-κB/IRF activation, leading to an inflammatory response. (Takeuchi & Akira 2010.)

Collectins. Collectins are soluble pattern recognition molecules and belong to C-type lectins. Five collectins have been recognised in humans. They have similar structures that include an N-terminal collagen-like region and a carboxyterminal recognition domain (CRD). Surfactant protein A (SP-A) and surfactant protein D (SP-D) collectins are produced and located in the lungs (Table 1). Recently, human collectin liver 1 and collectin placenta 1 were identified. Mannose-binding lectin (MBL) is a serum collectin but is also present in the upper airways (Table 1). Collectins recognise and bind carbohydrate structures on the pathogen surface and mediate innate immune responses by acting as opsonins for phagocytosis, by upregulating the expression of immune cell surface receptors and by acting as an activating ligand. In addition, MBL activates the complement lectin pathway. (Hickling *et al.* 2004, Pastva *et al.* 2007, Wright 2005.)

The levels of collectins are increased as an acute phase response to infectious agents or their components. Airway collectins specifically interact with a wide range of microorganisms and mediate the interactions with phagocytes. SP-A interacts with LPS and SP-D and MBL interact with oligosaccharide structures. Various studies have shown that SP-A and/or SP-D bind to respiratory pathogens, including influenza A virus (Hartshorn *et al.* 1997), RSV (Ghildyal *et al.* 1999, Hickling *et al.* 1999), *S. pneumoniae* (Hartshorn *et al.* 1998), *H. influenzae* (McNeely & Coonrod 1994) and Group A *Streptococcus* (Tino & Wright 1996). MBL has been reported to bind to influenza A virus (Hartshorn *et al.* 1993), *Neisseria meningitides* and *S. pneumoniae* (Neth *et al.* 2000).
Table 1. Description of human pattern recognition molecules.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Location</th>
<th>Target structures</th>
<th>Microbe interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toll-like receptors (TLRs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td>Plasma membrane</td>
<td>Triacyl lipopeptide</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR2</td>
<td>Plasma membrane</td>
<td>Peptidoglycan, lipopeptide</td>
<td>Gram-positive bacteria, fungi</td>
</tr>
<tr>
<td>TLR3</td>
<td>Endosome</td>
<td>dsRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR4</td>
<td>Plasma membrane</td>
<td>LPS</td>
<td>Gram-negative bacteria</td>
</tr>
<tr>
<td>TLR5</td>
<td>Plasma membrane</td>
<td>Flagellin</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR6</td>
<td>Plasma membrane</td>
<td>Dicacyl lipoprotein</td>
<td>Bacteria, viruses</td>
</tr>
<tr>
<td>TLR7/TLR8</td>
<td>Endosome</td>
<td>ssRNA</td>
<td>Viruses, bacteria</td>
</tr>
<tr>
<td>TLR9</td>
<td>Endosome</td>
<td>DNA</td>
<td>Viruses, bacteria</td>
</tr>
<tr>
<td>TLR10</td>
<td>Endosome</td>
<td>Not identified</td>
<td>Not identified</td>
</tr>
<tr>
<td>Non-toll-like receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD1 and -2</td>
<td>Cytoplasm</td>
<td>Peptidoglycan</td>
<td>Bacteria</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Cytoplasm</td>
<td>Short dsRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>MDA5</td>
<td>Cytoplasm</td>
<td>Long dsRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>Mannose receptor</td>
<td>Plasma membrane</td>
<td>Mannose</td>
<td>Bacteria, viruses and fungi</td>
</tr>
<tr>
<td>Dectin-1 and -2</td>
<td>Plasma membrane</td>
<td>β-glucan</td>
<td>Fungi</td>
</tr>
</tbody>
</table>

Collectins

| SP-A           | Lung           | LPS, carbohydrates, lipids        | Bacteria, viruses, fungi |
| SP-D           | Lung           | LPS, carbohydrates                | Bacteria, viruses, fungi |
| MBL            | Serum, lung    | Carbohydrates                     | Bacteria, viruses, fungi, yeast |

*ds/ssRNA, double-stranded/single-stranded ribonucleic acid; LPS, lipopolysaccharide; DNA, deoxyribonucleic acid; NOD, nucleotide-binding and oligomerisation domain; RIG, retinoic acid-inducible gene; MDA, melanoma differentiation-associated; SP, surfactant protein; MBL, mannose-binding lectin*

**Complement**

The complement system is an enzymatic cascade consisting of a complex group of more than 30 soluble plasma proteins and receptors. The complement plays a crucial role in innate defence mechanisms that mediate phagocytosis, cytolysis, inflammation, solubilisation of immune complexes and clearance of apoptotic cells. The complement also mediates indirect responses by promoting antibody responses. Because of their bacterial cell wall, complement-mediated opsonisation plays a more relevant role than cell lysis in the defence against Gram-positive bacteria. However, Gram-negative bacteria are sensitive to lysis as well as opsonisation by the complement. (Dunkelberger & Song 2010, Mold 1999, Rus et al. 2005.)
The complement can be activated in three different ways: the classical pathway, the lectin pathway or the alternative pathway. The classical pathway is activated when C1q, in complex with C1r and C1s serine proteases binds to the fragment crystallisable (Fc) region of antibodies attached to antigens on bacterial surfaces, viral envelopes and C-reactive protein (CRP). The lectin pathway is initiated by the binding of typical pattern recognition molecules, MBL or ficolins to carbohydrate groups on the surface of bacteria, yeast, viruses and parasites. Binding leads to the activation of MBL-associated serine proteases (MASPs). The alternative pathway is activated directly by the cell wall structures of microorganisms, including Gram-negative LPS, Gram-positive teichoic acids and viral membrane glycoproteins. Activation of all three pathways leads to a sequence of enzymatic reactions, including proteolytic cleavage of complement proteins and finally generation of the C3 convertase, which cleaves the central molecule of the cascade, C3. The C3b fragment combines with C3 convertase to form C5 convertase, which further cleaves C5. Cleaved complement fragments act as mediators of the host defences: opsonisation and clearance of pathogens by phagocytic cells (C3b and C4b fragments) and mediation of proinflammatory reactions (C3a, C4a and C5a fragments). C5b and complement protein C6-C9 form a membrane-attack complex (MAC), which assembles into the cell membrane and causes cell lysis. (Dunkelberger & Song 2010, Mold 1999, Rus et al. 2005.)

Deficiencies in the complement pathways may lead to impaired defence responses and increased susceptibility to infections and development of autoimmune diseases. Deficiencies usually have a heterogeneous genetic background, are rather rare and their prevalence varies in different ethnic groups. Deficiencies of C3 or proteins necessary to activate C3 are most strongly associated with infections. Usually they are associated with susceptibility to recurrent infections with encapsulated bacteria, including S. pneumoniae, N. meningitides and H. influenzae, and autoimmune diseases such as systemic lupus erythematosus and rheumatic arthritis. (Mold 1999, Sjöholm et al. 2006.)

Cytokines

Cytokines are small, non-structural proteins and important soluble mediators of cellular communication that regulates inflammation and immune responses. They are produced throughout the body from immune system cells. Specific microbial antigens stimulate host cells to secrete inflammatory cytokines which regulate cell
migration (chemotaxis) and the activities of T-cells, B-cells and phagocytes. The effects of cytokines are mediated by specific membrane receptors that bind their cytokine ligand and mediate signal transductions. Typical proinflammatory cytokines that promote inflammatory responses are interleukin (IL)-1α, IL-1β, IL-6, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. IL-8 is a typical chemokine, which regulates leukocyte migration. IL-4, IL-10 and IL-13 are anti-inflammatory cytokines that inhibit the proinflammatory cytokines and reduce inflammation. (Dinarello 2000, Haddad 2002.)

Cytokines in the respiratory tract are important mediators of the inflammatory response to infection. Cells in the respiratory airways release proinflammatory cytokines, including IL-6 and IL-8, when infected with rhinovirus, influenza A virus, adenovirus or RSV (Arnold et al. 1994, Bruder & Kovesdi 1997, Matsukura et al. 1996, Subauste et al. 1995). Furthermore, increased concentrations of cytokines like IL-1β, IL-6, IL-8 and TNF-α have been found in nasal secretions of children during upper respiratory tract infections (Noah et al. 1995). Increased IL-6 concentrations in nasal secretions have been associated with influenza A virus infection and with infection-related nasal symptoms (Gentile et al. 1998, Hayden et al. 1998, Skoner et al. 1999). In addition, increased IL-8 and IL-1 concentrations correlated with the rhinovirus-associated common cold (Proud et al. 1994, Turner et al. 1998). Thus, cytokines likely participate in the regulation of inflammation in respiratory tract infection.

2.1.2 Human gene polymorphisms in innate immunity associated with susceptibility to respiratory tract infections

Several genetic variations that are reported to cause susceptibility to respiratory tract infections have been found in molecules that are important in innate immune responses. Single-nucleotide polymorphisms (SNPs) in immune response genes may regulate the production of molecules that affect immune response and many of those polymorphisms predispose to an overall increased susceptibility to infections. Here I present some immunogenetic studies that have reported an association between both human gene polymorphisms in cytokines (Table 2) and pattern recognition molecules (Table 3) and respiratory tract infections.
Cytokine gene polymorphisms

Polymorphisms of genes that encode cytokines have been widely studied and reported to influence susceptibility to various infections (Table 2). The most studied cytokine SNPs in relation to respiratory infections are in the TNF-α and IL-6 genes.

The TNF-α gene is located at chromosome 6 in a region containing major histocompatibility complex genes and is thus highly polymorphic (Spies et al. 1986). A widely studied SNP of the TNF-α gene is the promoter polymorphism G/A at position -308 (SNP accession no. rs1800629). The A allele has been connected with increased TNF-α expression (Allen 1999) and with susceptibility to and the severity of infectious diseases. The A allele genotypes have been associated with chronic bronchitis in male adults (Huang et al. 1997) and chronic bronchitis and bronchopneumonia in the elderly (Cipriano et al. 2005). In recent studies among children, the A allele genotypes were associated with an increased risk of otitis media and a need for tympanostomy tubes, a marker of persistent or recurrent otitis media (Patel et al. 2006). In addition, the A/G and A/A genotypes were shown to cause an increased risk for upper respiratory tract infections and otitis media complicated by respiratory tract infection (Revai et al. 2009). The TNF-α -308A allele was also associated with chronic sinusitis with nasal polyposis (Bernstein et al. 2009). However, a negative association has also been reported (Mfuna Endam et al. 2010).

The IL-6 gene polymorphism -174G/C (rs1800795) has been associated with several inflammatory diseases (discussed in depth in chapter 2.4.1). Contradictory effects of the -174G/C polymorphism on IL-6 levels have been reported. In respiratory tract infection association studies, the C allele genotype has been linked to upper respiratory tract infections and otitis media in children (Emonts et al. 2007, Patel et al. 2006, Revai et al. 2009) and a more severe rhinovirus illness, measured as symptom scores, in adults (Doyle et al. 2010). Furthermore, the simultaneous presence of TNF-α -308 and IL-6 -174 polymorphic genotypes increased the risk for otitis media (Patel et al. 2006).

Doyle and co-workers (Doyle et al. 2010, Gentile et al. 2003) reported that IL-6 -174, IFN-γ 874 and TNF-α -308 polymorphisms affect the immune responses and illness severity of rhinovirus and RSV virus infections in adults. It has also been shown that IL-1α 4845 (rs17561), IL-1β -511 (rs16944) and TNF-α -308 and -238 polymorphisms are associated with nasal polyposis, a chronic
disease of sinusitis (Erlek et al. 2007). IL-1 α 4845 is also associated with chronic rhinosinusitis in an adult population (Mfuna Endam et al. 2010).

**Polymorphisms in genes that encode pattern recognition molecules**

In the TLR4 gene, there is an 896A/G SNP that causes a Asp/Gly change at position 299 (rs4986790) and an 1196C/T SNP that causes a Thr/Ile change at 399 (rs4986791). The TLR4 896 polymorphism has been shown to cause LPS hyporesponsiveness in humans (Arbour et al. 2000), and the 896G and 1196T alleles have been associated with susceptibility to invasive Gram-negative bacterial infections (Agnesi et al. 2002, Lorenz et al. 2002). TLR-4 SNPs were also associated with severe RSV bronchiolitis in infants (Tal et al. 2004).

A 2257G/A (753Arg/Gln) (rs5743708) polymorphism has been reported in the TLR2 gene. The polymorphism was associated with sepsis caused by Gram-positive staphylococcal bacteria (Lorenz et al. 2000), although a controversial result was reported after that (Moore et al. 2004). It was also shown that the A allele is associated with susceptibility to tuberculosis (Ogus et al. 2004) and, recently, also with recurrent respiratory tract infections in Turkish children (Kutukculer et al. 2007). However, the 2257G/A polymorphism was not associated with nasal polyposis or chronic rhinosinusitis in an adult population (Sachse et al. 2010, Tewfik et al. 2008).

There is a -159C/T (also referred as -260C/T, depending on whether the translation or transcription site is considered as site 0) (rs2569190) polymorphism in the promoter region of the CD14 gene. The polymorphism is located within the transcription site and may affect the transcription of the CD14 gene. The -159T allele genotype is associated with higher production of both soluble CD14 (Baldini et al. 1999) and membrane-bound CD14 receptor in monocytes (Hubacek et al. 1999). It has been reported that T allele genotypes are associated with C. pneumoniae seropositivity and infection (Eng et al. 2003, Rupp et al. 2004) (discussed in chapter 2.2.5). Compared to children with the C/C genotype, 12- to 24-month-old children with the T/T genotype had fewer acute otitis media episodes (Wiertsema et al. 2006b). This association was absent in older children. Children with the T/T genotype also had higher serotype-specific immunoglobulin G (IgG) response to pneumococcal conjugate vaccine. The results suggest that CD14 polymorphisms play an important role in the defence against otitis media in early childhood and in responsiveness to pneumococcal vaccine (Wiertsema et al. 2006b).
Table 2. Studies on the association between gene polymorphisms of cytokines and respiratory tract infections.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Study population</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>-308G/A</td>
<td>84 Taiwanese adults and 99 schoolchildren</td>
<td>A allele more common in patients with chronic bronchitis (p &lt; 0.001).</td>
<td>Huang et al. (1997)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-308G/A</td>
<td>73 Italian elderly</td>
<td>A allele more common in chronic bronchitis or bronchopneumonia (p = 0.034).</td>
<td>Cipriano et al. (2005)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-308G/A</td>
<td>384 U.S. children (mean age 4.9 yr.)</td>
<td>TNF-α G/A or A/A (OR 1.78, 95% CI 1.04-3.03) and IL-6 G/C or C/C (OR 1.75, 95% CI 1.07-2.85) associated with susceptibility to OM.</td>
<td>Patel et al. (2006)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-308G/A</td>
<td>241 U.S. children (age 6-35 mo.)</td>
<td>TNF-α G/A or A/A associated with increased risk for OM-complicating URI (OR 1.42, 95% CI 1.00-2.00). IL-6 G/C or C/C associated with increased risk for URI (IDR 1.24, 95% CI 1.00-1.54).</td>
<td>Revai et al. (2009)</td>
</tr>
<tr>
<td>IL-6</td>
<td>-174G/C</td>
<td>332 U.S. adults</td>
<td>A allele more common in patients with nasal polyposis (OR 1.85, 95% CI 1.4-3.09).</td>
<td>Bernstein et al. (2009)</td>
</tr>
<tr>
<td>IL-6</td>
<td>-174G/C</td>
<td>72 U.S. adults</td>
<td>IL-6 C/C genotype associated with increased magnitude of symptoms caused by rhinovirus (p &lt; 0.01) and IFN-γ A/A with rhinovirus seroconversion (p = 0.024). No association with IL-10 and TNF-α gene polymorphisms.</td>
<td>Doyle et al. (2010)</td>
</tr>
<tr>
<td>IL-6</td>
<td>-174G/C</td>
<td>29 U.S. adults</td>
<td>IL-6 C/C genotype associated with RSV symptoms, IFN-γ A/A with increased RSV-specific IgA (p = 0.02) and TNF-α G/A or A/A with increased RSV-specific IgG antibodies (p = 0.05). No association with IL-10 or TNF-β gene polymorphisms.</td>
<td>Gentile et al. (2003)</td>
</tr>
<tr>
<td>Gene</td>
<td>Polymorphism</td>
<td>Study population</td>
<td>Result</td>
<td>Reference</td>
</tr>
<tr>
<td>------</td>
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<td>-----------</td>
</tr>
<tr>
<td>IL-1α</td>
<td>4845G/T</td>
<td>188 Turkish adults</td>
<td>IL-1α G/T and T/T (p &lt; 0.05), IL-1β C/C (p = 0.01),</td>
<td>Erbek et al. (2007)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-511C/T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>-238G/A, -308G/A</td>
<td></td>
<td>genotypes associated with nasal polyposis.</td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>4845G/T</td>
<td>412 Canadian adults</td>
<td>IL-1α T allele associated with chronic rhinosinusitis</td>
<td>Mfuna Endeman et al. (2010)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-511C/T</td>
<td></td>
<td>(p = 0.02). No association with IL-1β or TNF-α.</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>-238G/A, -308G/A</td>
<td>and tagging SNPs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TNF, tumor necrosis factor; IL, interleukin; yr., year; OR, odds ratio; CI, confidence interval; OM, otitis media; mo., month; URI, upper respiratory infection; IDR, incidence density ratio; IFN, interferon; RSV, respiratory syncytial virus; Ig, immunoglobulin
Table 3. Studies on the association between gene polymorphisms of pattern recognition molecules and respiratory tract infections.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Study population</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>896A/G</td>
<td>189 infants (age &lt; 1 yr.)</td>
<td>896G and 1196T alleles associated with severe RSV bronchiolitis (p = 0.009).</td>
<td>Tal et al. (2004)</td>
</tr>
<tr>
<td>TLR2</td>
<td>2257G/A</td>
<td>143 Turkish children (mean age 5.2 yr.)</td>
<td>A allele associated with recurrent RTI (p &lt; 0.001).</td>
<td>Kutukculer et al. (2007)</td>
</tr>
<tr>
<td>TLR2</td>
<td>2257G/A and 10 other SNPs</td>
<td>406 Canadian adults</td>
<td>No association with chronic rhinosinusitis.</td>
<td>Tewfik et al. (2008)</td>
</tr>
<tr>
<td>TLR2</td>
<td>2257G/A</td>
<td>149 German adults</td>
<td>No association with nasal polyposis.</td>
<td>Sachse et al. (2010)</td>
</tr>
<tr>
<td>CD14</td>
<td>-159C/T</td>
<td>315 Chinese adults</td>
<td>T/T genotype associated with C. pneumoniae seropositivity (IgG titer ≥ 16) (OR 2.08, 95% CI 1.18-3.69).</td>
<td>Eng et al. (2003)</td>
</tr>
<tr>
<td>CD14</td>
<td>-159C/T</td>
<td>610 Caucasian coronary artery disease patients</td>
<td>CD14 -159T allele associated with chronic C. pneumoniae infection in peripheral blood monocytes (OR 1.7, 95% CI 1.08-2.65). No association with TLR-4 or TLR-2 polymorphisms.</td>
<td>Rupp et al. (2004)</td>
</tr>
<tr>
<td>TLR4</td>
<td>896A/G</td>
<td>2257G/A</td>
<td>T/T genotype was protective against recurrent OM (p = 0.004) in children aged 1-2 yr., but not in older children.</td>
<td>Wiertsema et al. (2006b)</td>
</tr>
<tr>
<td>MBL</td>
<td>Codons 52, 54 and 55</td>
<td>617 British children (age ≤ 18 yr.)</td>
<td>O¹ allele associated with infections (including RTI) requiring hospitalisation (OR 2.4, 95% CI 1.7-3.4).</td>
<td>Summerfield et al. (1997)</td>
</tr>
<tr>
<td>MBL</td>
<td>Codon 52, 54 and 55; -221X/Y</td>
<td>252 Greenlandic children (age &lt; 2 yr.)</td>
<td>MBL deficiency (XAO + O/O) associated with acute RTIs (OR 2.08, 95% CI 1.41-3.06).</td>
<td>Koch et al. (2001)</td>
</tr>
<tr>
<td>MBL</td>
<td>Codons 52, 54 and 55</td>
<td>413 Polish children (age 1-16 yr.)</td>
<td>O allele associated with recurrent RTIs (p = 0.01).</td>
<td>Cedzynski et al. (2004)</td>
</tr>
<tr>
<td>Gene</td>
<td>Polymorphism</td>
<td>Study population</td>
<td>Result</td>
<td>Reference</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
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<td>----------------------------</td>
</tr>
<tr>
<td>MBL</td>
<td>Codons 52, 54 and 55; -550H/L, -221X/Y, +4P/Q and 6 additional genetic variations</td>
<td>244 Dutch children (age 1-7 yr.)</td>
<td>Non-YA/YA associated with recurrent OM (p = 0.027).</td>
<td>Wiertsema et al. (2006a)</td>
</tr>
<tr>
<td>MBL</td>
<td>Codons 52, 54 and 55; -550H/L, -221X/Y, +4P/Q</td>
<td>190 Chinese children (age 0.5-6 yr.)</td>
<td>LXP haplotype and B allele associated with recurrent RTI (OR 1.63, 95% CI 1.01-2.62 and 1.94, 1.02-3.68).</td>
<td>Chen et al. (2009)</td>
</tr>
<tr>
<td>MBL</td>
<td>Codons 52, 54 and 55</td>
<td>9245 Danish adults</td>
<td>No association with RTI leading to hospitalisation.</td>
<td>Dahl et al. (2004)</td>
</tr>
<tr>
<td>MBL</td>
<td>Codon 54</td>
<td>236 Japanese adults</td>
<td>B allele associated with recurrent RTI (p = 0.003).</td>
<td>Gomi et al. (2004)</td>
</tr>
</tbody>
</table>

1 O stands for any of the exon 1 variant alleles B, C or D

TLR, toll-like receptor; yr., year; RSV, respiratory syncytial virus; RTI, respiratory tract infection; Ig, immunoglobulin; OR, odds ratio; CI, confidence interval; OM, otitis media; MBL, mannose-binding lectin
2.2 *Chlamydia pneumoniae*

In 1985, an unusual strain of *Chlamydia psittaci* was found in an epidemic of mild pneumonia that had occurred in northern Finland in 1978 (Saikku *et al.* 1985). Later, the strain was isolated from adults with acute respiratory tract infections and was called *C. psittaci* strain TWAR (Grayston *et al.* 1986). The strain TWAR was identified as a new species of the genus *Chlamydia*, and named *Chlamydia pneumoniae* (Grayston *et al.* 1989).

According to the new taxonomy, *Chlamydia* should be divided into two genera, *Chlamydia* and *Chlamydophila*, where *C. pneumoniae* belongs to the latter (Everett *et al.* 1999). However, the new taxonomy has not been generally accepted and *Chlamydia pneumoniae* is more widely used than *Chlamydophila pneumoniae* (Schachter *et al.* 2001, Stephens 2008). *C. pneumoniae* and *C. trachomatis* are common human pathogens, whereas the other species occur mainly in animals.

2.2.1 Pathogenesis

*C. pneumoniae* development cycle

*C. pneumoniae* is a Gram-negative, obligate intracellular bacterium and it has a unique biphasic development cycle. The cycle starts when the extracellular infectious forms, elementary bodies (EB), are attached to the host cell and phagocytosed into the cell. Inside the cell the EBs prevent the fusion of phagosome with lysosome by remaining in the intracellular vacuole and transform into larger replicating forms, called reticulate bodies (RB). The RBs replicate using host cell energy resources and form characteristic inclusions. At the end of the cycle the RBs transform back into EBs, and finally the infectious EBs are released to the cytoplasm. (Hogan *et al.* 2004.)

Certain factors such as antibiotics, IFN-γ or depletion of certain essential nutrients may lead to the formation of abnormal RBs. These nonreplicative and noninfectious, but viable, structurally atypical chlamydial forms may persist in inclusions inside the host cell for a long time. Persistent *C. pneumoniae* is able to escape the host’s defence mechanisms and has proved to be untreatable with common antibiotics (Gieffers *et al.* 2001). Infection studies of cell cultures have shown that *C. pneumoniae* becomes spontaneously persistent in monocytes (Airenne *et al.* 1999). Persistent infection may persistently activate the host cell
signalling pathways and thus induce inflammation and play a role in the pathogenesis of different kinds of inflammatory diseases, including atherosclerosis. (Hogan et al. 2004, Kern et al. 2009.)

**C. pneumoniae structural components important in pathogenesis**

The structural components of *C. pneumoniae* - LPS and heat shock proteins (especially Hsp60) - are important inducers of immune response in chlamydial pathogenesis. LPS is an endotoxin, found in the outer membrane of Gram-negative bacteria, that activates macrophages and endothelial cells and induces the production of proinflammatory cytokines. The structure of chlamydial LPS resembles the rough form of enterobacterial LPS, and studies have shown that chlamydial LPS has much lower endotoxin activity than does enterobacterial LPS. Weak endotoxin activity possibly leads to a weak host immune response, enabling persistent infection. (Ingalls et al. 1995, Tsutsumi-Ishii et al. 2008.)

Hsps are highly conserved proteins that are produced by all organisms. Under physiological conditions they act as molecular chaperons for proteins, whereas their expression is increased in response to cellular stress. Hsps act as important antigens that induce the immune system. Bacterial Hsps have high homology with their human counterparts and therefore can act as antigens in the autoimmune inflammatory process. Chlamydial Hsp60 is an important antigen in chronic *C. pneumoniae* infection, and antibody response to chlamydial Hsp60 has been associated with atherosclerosis (Huittinen et al. 2002). In addition, both human and chlamydial Hsp60 proteins have been found to localise in atherosclerotic plaques (Kol et al. 1998). Chlamydial Hsp60 has also been shown to induce oxidation of low-density lipoprotein (LDL) in monocytes (Kalayoglu et al. 1999).

**Role of C. pneumoniae in the pathogenesis of vascular diseases**

The role of *C. pneumoniae* in the pathogenesis of vascular diseases has been studied widely and different mechanisms have been proposed for how *C. pneumoniae* infection may promote atherosclerosis. From the infection site in the lungs, *C. pneumoniae* may disseminate within infected bronchoalveolar macrophages into the circulation and vascular tissue (Moazed et al. 1998). *C. pneumoniae*-infected monocytes are able to adhere to arterial endothelial cells (Kalayoglu et al. 2001) and infect and multiply within endothelial cells and smooth muscle cells (Gaydos et al. 1996). *C. pneumoniae* is able to induce human
macrophage foam cell formation (Kalayoglu & Byrne 1998a) through a process where chlamydial LPS causes cholesterol uptake from LDL (Kalayoglu & Byrne 1998b). The presence of persistent *C. pneumoniae* in atherosclerotic plaque may promote a local inflammation response: induction of cytokine production and thus changes in lipid metabolism.

*C. pneumoniae* particles in atherosclerotic tissue and lesions have been verified by different methods (Jackson *et al.* 1997, Juvonen *et al.* 1997, Lajunen *et al.* 2008b, Ramirez 1996). In addition, animal models have shown direct evidence of the role of *C. pneumoniae* infection in the atherosclerosis process (Laitinen *et al.* 1997). Also primary antibiotic treatment studies have reported positive results in the prevention of cardiovascular events (Gupta *et al.* 1997, Wiesli *et al.* 2002), although this effect was not detected in large-scale antibiotic treatment studies (Cannon *et al.* 2005, Grayston *et al.* 2005, O'Connor *et al.* 2003).

### 2.2.2 Seroepidemiology

A microimmunofluorescence (MIF) test is a specific and sensitive serological test to detect *C. pneumoniae* antibodies from a serum sample and to differentiate between current and previous infections (Dowell *et al.* 2001, Paldanius *et al.* 2003, Wang 2000). In a primary *C. pneumoniae* infection, IgM antibodies appear about 3 weeks and IgG and IgA antibodies not until 6–8 weeks after the infection. The IgM titres fall in 2 to 6 months, but IgG may persist for several years. In reinfection, the IgM response may not develop at all or may appear only at low titres. However, the IgG and IgA titres rise quickly, often in 1–2 weeks after infection. It has been shown that IgG and IgA antibodies decrease rapidly after an acute infection and persist longer after reinfections than after primary infections. Therefore, antibody persistence is seen more often in older persons than in younger ones. (Grayston *et al.* 1990, Kuo *et al.* 1995, Paldanius *et al.* 2005.)

Seroprevalence increases with age. The infection is most common among school-aged children, and seroprevalence is about 50% by the age of 20 years and 70–80% in elderly people. High seroprevalence in older age might be due to reinfections and persistent infections. In an adult population, seroprevalence has been shown to be higher in men than in women and in smokers than in non-smokers (Karvonen *et al.* 1994). (Kuo *et al.* 1995, Saikku 1992.)
2.2.3 *C. pneumoniae* in respiratory tract infections

*C. pneumoniae* is a common cause of upper and lower respiratory tract infections, such as acute pneumonia, bronchitis, sinusitis and pharyngitis, throughout the world. About 10% of pneumonia and 5% of bronchitis and sinusitis cases are caused by *C. pneumoniae*. In Finland, *C. pneumoniae* has been shown to be the etiological agent in 10% of community-acquired pneumonia cases among adults (Jokinen et al. 2001) and in 43% of hospitalised patients during a *C. pneumoniae* epidemic (Kauppinen et al. 1995). Most infections are asymptomatic or mildly symptomatic illnesses, and severe forms are diagnosed particularly in elderly patients and those with chronic diseases. Pharyngitis, cough and transient fever are common characteristics in *C. pneumoniae* pulmonary infections. Primary infection occurs mostly in schoolchildren and reinfections and reactivation particularly in elderly people. *C. pneumoniae* epidemics have been reported in closed population groups, like in garrison situations (Ekman et al. 1993). Epidemics occur in all seasons of the year, and in Finland epidemics occur at intervals of 4 to 5 years (Paldanius et al. 2005). (Grayston et al. 1990, Kuo et al. 1995, Saikku 1992.)

*C. pneumoniae* has a tendency to persist and cause chronic infections. Persistent infections have been associated with several diseases.

2.2.4 Persistence of *C. pneumoniae* antibodies – a marker of a chronic infection?

There are no accepted serologic criteria for chronic *C. pneumoniae* infection. However, persistence of IgG antibodies or the presence of IgA antibodies has been used to indicate persistent or chronic infection. The presence of IgG antibodies is a marker of previous exposure to *C. pneumoniae*. Due to their short half-life, IgA antibodies disappear faster than IgG after seroconversion, and therefore the presence of IgA antibodies has been suggested to be a better marker of chronic infection than are IgG antibodies. (Dowell et al. 2001, Grayston et al. 1990, Paldanius et al. 2005.) It has been demonstrated that already 7- to 8-year-old children have persistently elevated *C. pneumoniae* IgG and IgA antibodies, suggesting that chronic *C. pneumoniae* infections may exist already in childhood (Volanen et al. 2003).

Persistent *C. pneumoniae* infection has been associated with several disease states that are often chronic in nature, including asthma and chronic obstructive

**C. pneumoniae and asthma**

The association between *C. pneumoniae* and asthma has been widely studied recently. However, the causative role of *C. pneumoniae* in asthma has not been resolved yet. Hahn *et al.* (1991) reported a relationship between a positive *C. pneumoniae* titre and wheezing and a diagnosis of asthmatic bronchitis in adult patients and suggested *C. pneumoniae* as a possible cause of asthma. After that, especially a correlation between previous and/or chronic *C. pneumoniae* infection and chronic asthma in adults has been reported (Agarwal & Chander 2008, Biscione *et al.* 2004, Cook *et al.* 1998, Gencay *et al.* 2001, Huittinen *et al.* 2001, von Hertzen *et al.* 1999, von Hertzen *et al.* 2002). Studies have hypothesised that asthmatic individuals may be more susceptible than normal individuals to *C. pneumoniae* infection, possibly because of impaired immune response in asthma (Biscione *et al.* 2004). On the other hand, it has also been suggested that *C. pneumoniae* infections may play a role in the pathogenesis of asthma and can induce acute asthma exacerbations (Blasi 2004). Chronic *C. pneumoniae* infection may also lead to an increase in the severity of asthma by contributing to chronic inflammation and airway hyperresponsiveness (Black *et al.* 2000). In a recent study among military recruits, *C. pneumoniae* IgG seroprevalence was higher in conscripts with asthma than in those without, and furthermore, IgG and IgA antibodies persisted more often in asthmatic conscripts than in nonasthmatic ones (Paldanius *et al.* 2007). It was also shown in the same conscript population that prolonged *C. pneumoniae* infections were more common among men with asthma than among those without (Juvonen *et al.* 2008a). Those results suggested that asthmatic persons are more susceptible to *C. pneumoniae* infections, especially to prolonged or chronic infections.

**C. pneumoniae and cardiovascular diseases**

Several studies have reported an association between *C. pneumoniae* and atherosclerosis and cardiovascular diseases e.g. myocardial infarction, coronary heart disease (CHD), transient ischemic attack, abdominal aortic aneurysm, valvular lesions and stroke. Chronic *C. pneumoniae* infections may induce the
atherosclerotic process, whereas acute infections may trigger acute cardiovascular events. (Leinonen & Saikku 2002, Saikku 1999, Saikku 2002.) C. pneumoniae infection was first associated with CHD and myocardial infarction in a seroepidemiologic study in 1988 (Saikku et al. 1988). After that, several studies have confirmed the association between elevated C. pneumoniae antibodies and cardiovascular diseases (Danesh et al. 2002, Danesh et al. 2000b, Saikku et al. 1992, Watson & Alp 2008). In addition, circulating immune complexes containing chlamydial LPS and antibodies to chlamydial proteins have been detected in cardiovascular disease patients (Leinonen et al. 1990, Linnanmäki et al. 1993). Immune complexes containing chlamydial antigens may be transported directly into the blood circulation and thus have been suggested to be a good marker of intravascular chronic infection. In addition, persistent C. pneumoniae seropositivity has been associated with increased aortic intima-media thickness already in 11-year-old healthy children (Volanen et al. 2006). That study suggested that C. pneumoniae may play a role in the pathogenesis of early atherosclerosis.

Several studies have shown that IgA antibodies, especially when persistently elevated, predict cardiovascular diseases better than IgG antibodies (Danesh et al. 2002, Huittinen et al. 2003, Roivainen et al. 2000). It is also possible that elevated levels of C. pneumoniae antibodies alone are not a significant risk factor for cardiovascular diseases. The presence of C. pneumoniae antibodies accompanied by markers of systemic inflammation and autoimmunity might be a better indication of a chronic infection and thus a stronger predictor of coronary events. It has been shown that a combination of C. pneumoniae seropositivity and slightly elevated CRP levels increased the risk for CHD substantially compared with the presence of seropositivity alone (Roivainen et al. 2000). In addition, the coronary risk was significant when persistently elevated antibodies to C. pneumoniae and autoimmune antibodies to human Hsp60 were present in sera, and the risk further increased considerably when present together with elevated CRP levels (Huittinen et al. 2003).

C. pneumoniae infection may also have an influence on lipid metabolism that is recognised in the pathogenesis of atherosclerosis. TNF-α, IL-1, IL-6 and other acute-phase cytokines mediate changes in lipid metabolism (Gallin et al. 1969). The cytokines further lead to an increase in serum triglyceride and total cholesterol levels (Feingold & Grunfeld 1987). LPS is an efficient inducer of cytokines and has been shown to cause a decline in high-density lipoprotein (HDL) cholesterol levels (Cabana et al. 1989). In a Finnish male population, C.
pneumoniae IgG seropositivity (Laurila et al. 1997a) and persistently elevated IgG and IgA antibodies over a 3-year period (Laurila et al. 1997b) were associated with elevated serum triglyceride and decreased HDL cholesterol concentrations. *C. pneumoniae* has been shown to induce production of TNF-α, IL-1 and IL-6 in blood mononuclear cells (Kaukoranta-Tolvanen et al. 1996). Possibly, chronic *C. pneumoniae* infection may cause continuous low-level cytokine production in atherosclerotic lesions and thus lead to an atherogenetic lipid profile. In addition, *Helicobacter pylori* infection has also been associated with elevated serum triglyceride and total cholesterol concentration and CHD, as well (Laurila et al. 1999, Patel et al. 1995). An altered lipid profile leads to obesity and metabolic syndrome, which are connected with CHD in several studies. Studies have also associated *C. pneumoniae* infection with elevated fibrinogen levels, slightly elevated CRP concentration and hypertension, which all are well recognised risk factors for CHD (Leinonen 2000).

### 2.2.5 Human gene polymorphisms in innate immunity associated with chlamydial infections

**Immunogenetics associated with *C. pneumoniae***

Only a few studies on polymorphisms in human genes of innate immunity that affect susceptibility to *C. pneumoniae* infection have been published. The promoter polymorphism (C/T) at position -159 in the *CD14* gene, which is associated with increased levels of CD14 (Hubacek et al. 1999, Karhukorpi et al. 2002), has been associated with *C. pneumoniae* infection. Eng et al. reported that the T allele genotype was associated with an increased risk for *C. pneumoniae* seropositivity (Eng et al. 2003), and TNF-α production in whole blood cell culture after stimulation by *C. pneumoniae* has been shown to be significantly higher in subjects with the T/T genotype compared with those with the C/C genotype (Eng et al. 2004). Recently, the T/T genotype was shown to increase *C. pneumoniae* growth in human macrophages (Poikonen et al. 2009). In addition, the T/T genotype was significantly associated with chronic *C. pneumoniae* infection of blood monocytes in coronary artery disease patients (Rupp et al. 2004). The polymorphism regulates CD14 expression on monocytes and thus, may be responsible for susceptibility to bacterial infections. It has been speculated that a higher response to LPS in affected subjects could lead to an increased
inflammatory reaction, which may promote atherogenesis (Eng et al. 2003). A few studies have reported an association between the T allele genotype and cardiovascular disease (Hubacek et al. 1999, Shimada et al. 2000, Unkelbach et al. 1999). Furthermore, persistent C. pneumoniae infection was a significant risk factor for stroke in patients with the CD14 C/C genotype, suggesting that the CD14 polymorphism may affect the risk of stroke by influencing susceptibility to C. pneumoniae infection (Lin et al. 2008).

Polymorphisms in the TLR2, TLR4 and LBP genes have been studied, but no association with C. pneumoniae has been reported (Poikonen et al. 2009, Rupp et al. 2004).

Immunogenetics associated with C. trachomatis

Several immunogenetic association studies in relation to C. trachomatis have been reported. The studies have particularly concentrated on genetic variations of genes important in innate immunity, including pathogen recognition molecules and cytokines that have been under interest also in immunogenetic studies of C. pneumoniae. Often the studies have observed a risk of developing C. trachomatis-induced late complications, e.g. tubal infertility, which are often a consequence of repeated and persistent infections and an induced host immune response - also common in C. pneumoniae infections. (Morre et al. 2009.)

SNPs in the TLR4 (896A/G), TLR9 (-1237T/C, 2848G/A), CD14 (-159C/T) and NOD2 genes have not been associated with C. trachomatis infection or tubal pathology in single gene analyses. However, an increased risk for C. trachomatis was observed to induce tubal damage in Dutch women carrying at least two SNPs in these genes (den Hartog et al. 2006). A structural polymorphism in the MBL2 gene (codon 54, B allele) has been associated with tubal occlusions in a Caucasian population, and the risk was higher in women who were seropositive than in those who were seronegative for C. trachomatis (Sziller et al. 2007). (Morre et al. 2009.)

The most interesting findings of cytokine gene polymorphism studies have been reported for anti-inflammatory cytokine IL-10. The IL-10 -1082 A/A genotype, which causes low IL-10 expression, has been associated with tubal factor infertility (Kinnunen et al. 2002). In a recent study by Öhman et al. (2009), the IL-10 -1082A allele and TNF-α -308A allele were risk factors for severe C. trachomatis-associated tubal damage. In addition, IL-10 SNPs have been reported to increase the risk of complications of trachoma in several studies, but with
contradictory results. No association has been found between SNPs in the \( IL-1B, \) \( IL-2, \) \( IL-4 \) or \( IL-6 \) genes and tubal damage or \( C. \) \textit{trachomatis} infection. SNPs in the \( TNF-\alpha \) and \( IFN-\gamma \) genes have been associated with trachoma complications in a few studies. (Morre \textit{et al.} 2009.)

2.3 Mannose-binding lectin (MBL)

Mannose-binding lectin (MBL), also known as mannan-binding lectin or mannos-binding protein, is a circulating C-type lectin and a member of the collectin family that is able to recognise sugar structures on the surfaces of a wide range of microorganisms. MBL is synthesised in the liver and regulated as part of the acute-phase response in innate immunity. Although MBL is a serum protein, it is also detected, for example, in the upper respiratory airways (Garred \textit{et al.} 1993). Recently MBL was shown to be present in the airways of children with lower respiratory infection but was undetectable in children without infection (Fidler \textit{et al.} 2009). Thus, MBL may play a role in local pulmonary host defence, too.

2.3.1 The structure of MBL

MBL has a structure characteristic of the collectin family, possessing carbohydrate recognition domains (CRD) and collagenous regions. MBL protein consists of three to six trimers, which all are composed of three identical peptide chains. Each trimeric subunit consists of a C-terminal cluster of three CRDs, a hydrophobic neck region, a collagenous triple helix and a disulphide-linked N-terminal region. Each CRD binds a calcium ion and is then able to make coordination bonds with the 3- and 4-hydroxyl groups of mannose and other selected sugar residues such as N-acetyl-D-glucosamine, N-acetyl-mannosamine, fucose and glucose. The collagenous regions interact with a serine protease called MBL-associated serine proteases MASP-1, MASP-2 and MASP-3, assembling MBL-MASP complexes, which have the ability to activate the complement (Dahl \textit{et al.} 2001, Matsushita & Fujita 1992). (Ip \textit{et al.} 2009, Turner 1996, 2003.)

2.3.2 The functions of MBL

MBL is a pattern recognition molecule and has the ability to selectively recognise specific carbohydrate structures of infectious agents and apoptotic cells. The multimeric structure of MBL allows it to make high-avidity bindings with
carbohydrate structures of ligands such as mannan, peptidoglycans, teichoic acid and lipoglycans of Gram-negative and Gram-positive bacteria, mycobacterium, yeasts, viruses and fungi. MBL is able to make strong bindings with different Candida species, S. aureus and beta-hemolytic group A Streptococcus and low or heterogeneous bindings with S. pneumoniae, H. influenzae, N. meningitides, Escherichia coli and Klebsiella species (Jack & Turner 2003, Neth et al. 2000, van Emmerik et al. 1994). As an example, MBL is able to bind with teichoic acid and peptidoglycan from Gram-positive bacterium S. aureus (Ip et al. 2008, Nadesalingam et al. 2005). There is a strain variation within bacterial species in MBL binding and thus the findings between studies have been inconsistent. In addition, MBL was shown to bind intracellular bacteria, including different Chlamydia species (Swanson et al. 1998) and Legionella pneumophila (Kuipers et al. 2003), and certain viruses, including influenza A (Hartshorn et al. 1993, Kawai et al. 2007), human immunodeficiency virus (HIV) (Ji et al. 2005), herpes simplex virus 2 (Fischer et al. 1994) and severe acute respiratory syndrome-coronavirus (Ip et al. 2005). Recently, it was shown that MBL cooperated with TLR2 in responding to S. aureus, suggesting that microbial ligands may be recognised simultaneously by multiple receptors (Ip et al. 2008). As most of the sugar targets of MBL are not normally exposed on mammalian cell surfaces at high densities, MBL does not usually recognise self-structures. (Ip et al. 2009.)

MBL protein has an important role in first-line host defence as an acute-phase reactant after exposure to an infectious organism. MBL is able to activate the complement via the lectin pathway through MASPs. The activation of MBL-MASP complexes leads to the formation of C3 convertase, which is indistinguishable from the C3 convertase of both classical and alternative pathways. Produced complement fragments act as an opsonin for phagocytosis and as mediators of inflammation. MBL is also able to act as an opsonin directly by agglutinating micro-organisms, leading to their clearance by collectin receptor-mediated phagocytosis. MBL also has a major role in the modulation of inflammation. An in vitro whole blood culture where N. meningitides was incubated with increasing concentrations of MBL showed that MBL at a low concentration (< 4000 ng/ml) enhanced the release of proinflammatory cytokines TNF-α, IL-1β and IL-6, whereas MBL at a higher concentration suppressed cytokine release (Jack et al. 2001). In another study, S. aureus peptidoglycan increased the secretion of proinflammatory cytokines from monocytes but MBL-peptidoglycan complex inhibited the production of cytokines other than the production of chemokine IL-8 (Nadesalingam et al. 2005). These studies suggest
that MBL may also be able to reduce phagocyte-mediated inflammation. (Ip et al. 2009, Turner 2003.)

2.3.3 Human MBL2 gene polymorphisms

There are two human MBL genes, but MBL1 is a pseudogene and only MBL2 encodes a protein product. The MBL2 gene is located at chromosome 10q11.2-q21 (Sastry et al. 1989) and contains four exons interrupted by three introns (Taylor et al. 1989) (Fig. 1). Exon 1 encodes for the signal peptide, a cysteine-rich domain and a part of the Gly-Xaa-Yaa repeats of the collagenous region, exon 2 encodes the remaining Gly-Xaa-Yaa repeats, exon 3 the neck region and exon 4 the CRD. The promoter region contains several consensus elements that probably regulate gene expression. (Garred 2008.)

Previous studies have indicated that SNPs in the coding sequence and promoter region of the MBL2 gene have a strong effect on the MBL protein structure and the serum concentration of MBL. Three of these SNPs are located in exon 1 at codons 52 (CGT → TGT) (rs5030737) (Madsen et al. 1994), 54 (GGC → GAC) (rs1800450) (Sumiya et al. 1991) and 57 (GGA → GAA) (rs1800451) (Lipscombe et al. 1992). The wild-type alleles of these polymorphisms are designated as A, and the other alleles are known as D, B and C, respectively. These variant alleles are often pooled and called the O allele. All these structural mutations impair the oligomerisation of MBL protein, leading to a profoundly reduced serum level and function of MBL. Individuals who are heterozygous for any of the variant alleles have been reported to have reduced levels of MBL protein, and individuals with homozygosity or a combination of the variant alleles have virtually no circulating MBL in their serum. The effect of the D allele is less significant than that of the B or C alleles. (Garred 2008, Garred et al. 2006.)

The frequencies of these variant alleles vary between different ethnic groups. B allele occurs with high frequency in Caucasians and Asians but is rare in the Sub-Saharan African population. In contrast, the C allele is rare in Europeans and absent among Asians but is very common in Sub-Saharan Africans. The D allele is rare in all populations. (Crosdale et al. 2000, Garred 2008, Garred et al. 2006.) In the Finnish population, the proportions of structural variant genotypes have been described to be similar to those in the Caucasian population, the B allele being the most common variant allele (Aittoniemi et al. 2005).
The promoter 1 region is also highly polymorphic, and three polymorphisms are associated with different MBL levels independent of the exon 1 variant alleles. Two of these SNPs are situated in the 5′ regulatory region at positions -550 (alleles H and L) (rs11003125) and -221 (alleles X and Y) (rs7096206) and one in the 5′ untranslated region at position +4 (alleles P and Q) (rs7095891) (Madsen et al. 1995, Madsen et al. 1998a). The promoter mutations affect transcriptional activity, which results in reduced levels of MBL protein. Haplotypes HY, LY and LX have been correlated with high, medium and low levels of MBL protein, respectively (Madsen et al. 1995). Of all the promoter mutations, HYP is associated with the highest levels of MBL, LYQ and LYP with intermediate levels and LXP with the lowest level of MBL (Madsen et al. 1998a).

The promoter polymorphism sites are in linkage disequilibrium with the exon mutations, and seven common haplotypes - HYPA, HYPD, LYPA, LYPB, LYQA, LYQC and LXPA - have been identified in different populations. In recent studies, some additional polymorphisms have been described that could improve the correlation between serum MBL levels and MBL2 variations (Bernig et al. 2005, Bernig et al. 2004, Wiertsema et al. 2006a).

![SNPs within the promoter 1 and exon 1 regions of the MBL2 gene. Modified after Matsushita et al. (2001).](image)

**Fig. 1.** SNPs within the promoter 1 and exon 1 regions of the MBL2 gene. Modified after Matsushita et al. (2001).
2.3.4 The association between MBL deficiency and infections

The serum MBL levels of healthy people vary between individuals and different ethnic groups, ranging from 0 ng/ml to over 5000 ng/ml, depending on genetic variations and age. A typical median MBL concentration was 1420 ng/ml in Chinese blood donors (Ip et al. 2004), 1550 ng/ml in Australian blood donors (Minchinton et al. 2002), 2800 ng/ml in healthy Turkish children (Uguz et al. 2005), 992 ng/ml in Danish blood donors (Garred et al. 1992) and 4002 ng/ml in Finnish adult persons (Aittoniemi et al. 1996). The MBL level seems to reach its maximum within 1 month after birth and it declines during childhood and adulthood (Aittoniemi et al. 1996, Terai et al. 1993). The MBL concentration increases up to 1.5- to 3-fold during an acute phase reaction (Thiel et al. 1992). However, individuals with an MBL2 variant allele genotype appear to be unable to achieve as high MBL levels as those possessing a wild-type genotype.

MBL deficiency is caused by structural variant alleles in exon 1 of the MBL2 gene. MBL deficiency is usually defined by the lower detection level of the assay used or by the presence of a homozygous structural variant allele genotype. MBL deficiency has been reported in 5% or more of the general population (depending on the cut-off concentration used) (Aittoniemi et al. 1996, Turner 1991). Thus, it is the most common primary human immunodeficiency in many populations.

MBL deficiency is recognised as a functional defect in opsonisation and phagocytosis. It leads to increased susceptibility to several kinds of infectious and autoimmune diseases. Initially, an opsonisation defect was linked with low levels of MBL in children (Super et al. 1989). After that, several studies have been done in paediatric populations and MBL structural variant alleles have been shown to be associated with severe infections (Summerfield et al. 1997) and acute respiratory tract infections (Koch et al. 2001). It was suggested that MBL may be especially important during early childhood, particularly between 6 to 18 months of age, when the child's own adaptive immune response is still immature. Nevertheless, studies among adults have also found an association between MBL deficiency and infections (Kakkanaiah et al. 1998, Summerfield et al. 1995).

However, a majority of individuals with MBL deficiency are healthy, and studies indicating no association with infections have been reported (Dahl et al. 2004). One hypothesis is that MBL deficiency is relevant only in individuals with another coexisting immune deficiency or when their immune system is compromised some other way. MBL deficiency is a significant risk factor for infections, for example, in the context of IgG and/or IgA subclass deficiency.

A number of recent studies have reported associations between MBL and different infections and diseases, but only part of them can be referred to here. MBL deficiency has been associated with susceptibility to HIV-1 infection (Garred et al. 1997, Tan et al. 2009), herpes simplex virus 2 infection (Seppänen et al. 2009), hepatitis B and C infections (Brown et al. 2007), severe bacterial infections, e.g. meningococcal and pneumococcal infections and sepsis (Eisen et al. 2008, Hibberd et al. 1999, Roy et al. 2002), pulmonary tuberculosis (Capparelli et al. 2009), parasitic infections and fungal infections (Eisen & Minchinton 2003). Huttunen et al. (2008) recently reported that smoking was a significant risk factor for Gram-positive bacteraemia in patients carrying the MBL2 O allele, but the O allele was not associated with an overall risk of bacteraemia or mortality. The study suggested that smoking possibly favours phenotypic expression of MBL insufficiency. Jounio et al. (2010) showed that an MBL level below the median level was a significant risk factor for carriage of oropharyngeal β-hemolytic streptococci and N. meningitides only in nonsmokers but not in smokers. The study also showed that low-MBL-producing haplotypes may be associated with carriage of these bacteria. The hypothesis was that increased bacterial colonisation of smokers may obscure the effect of MBL.

MBL may also modulate disease severity, which may be related to the ability of MBL to regulate the proinflammatory cytokine response. Chronic inflammation caused by MBL deficiency may thus be a risk factor for atherosclerosis and myocardial infarction (Best et al. 2004, Jowett 2010, Madsen et al. 1998b, Saevarsdottir et al. 2005). However, contradictory results have also been presented: high serum MBL levels and MBL2 genotypes that determine high concentrations have been associated with cardiovascular diseases, suggesting a theory of MBL-induced persistent inflammation, e.g. through complement activation (Keller et al. 2006, Pesonen et al. 2009). MBL deficiency may also predispose to cardiovascular diseases through susceptibility to infections. Individuals with MBL2 O alleles and positive serum antibodies to C. pneumoniae may be more likely to have cardiovascular diseases (Rugonfalvi-Kiss et al. 2002). (Dommett et al. 2006, Eisen & Minchinton 2003.)

It has also been proposed that MBL deficiency leads to certain autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis (Tsutsumi
2005) and recently, also type 1 diabetes (Araujo et al. 2007, Tsutsumi et al. 2003). The possible explanation between MBL deficiency and autoimmunity is that MBL has an important role in the clearance of potential autoantigens, including apoptotic cells and DNA.

Studies have also been conducted on the association between MBL deficiency and respiratory tract infections. There are several studies on MBL2 variants and respiratory tract infection susceptibility among children, and most of them identify the A/O and O/O genotypes as risk factors for infections (Cedzynski et al. 2004, Chen et al. 2009, Koch et al. 2001, Summerfield et al. 1997, Wiertsema et al. 2006a). Recently, it was shown that MBL levels were significantly lower in children with recurrent respiratory tract infections compared with healthy controls (Chen et al. 2009). Earlier studies have also reported that MBL levels did not differ between children with repeated respiratory tract infections and healthy individuals (Aittoniemi et al. 1998, Thorarinsdottir et al. 2005). However, the latter study found that sustained low levels of MBL were associated with recurrent otitis media. In adults, a few studies on the association between MBL2 polymorphisms and respiratory tract infections have reported contradictory results. Gomi et al. (2004) found the structural variant allele B to be a risk factor for recurrent respiratory tract infections among adult patients in a hospital-based study. On the other hand, Dahl et al. (2004) found no association between MBL2 polymorphisms and respiratory tract infections among adults in a large population-based study. A recent study in an adult Caucasian population reported that MBL deficiency (defined as \( \leq 50 \) ng/ml) was significantly associated with recurrent and/or severe infections, including respiratory tract infections, and the association was independent of concomitant immunodeficiency (Hoeflich et al. 2009). Studies on the association between MBL2 polymorphisms and respiratory tract infections are presented in Table 3. (Ruskamp et al. 2006.)

### 2.3.5 MBL in the pathogenesis of chlamydial infection

Chlamydiae are known to enter the host cell by ligand-receptor-mediated endocytosis. Several studies have investigated the structure of the carbohydrates of the outer membrane of chlamydia and their role in the infectivity of host cells. Swanson and Kuo (1991) reported that the carbohydrates in glycoproteins are mannose, galactose, fucose and N-acetyl glucosamine. Later they showed that the carbohydrate moieties of the major outer membrane protein of chlamydia are involved in the attachment and infectivity of the organisms to HeLa cells.
(Swanson & Kuo 1994). Furthermore, Kuo et al. (1996) showed that major outer membrane protein is glycosylated with high mannose oligosaccharides and they indicated that these structures mediate attachment and internalisation of the organisms to the host cell and can inhibit infectivity. Previous studies have suggested that exogenous oligosaccharide ligands, e.g. mannan and mannose 6-phosphate (M6P), could inhibit the infectivity of *C. trachomatis* and *C. pneumoniae*, respectively (Kuo et al. 2007, Puolakkainen et al. 2005). Those studies also suggested that *C. pneumoniae* uses the M6P receptor, while *C. trachomatis* uses the mannose receptor for infection. In addition, *in vitro* studies by Swanson et al. (1998) have shown that MBL may play a role in protecting against chlamydia by inhibiting infection of the host cells. They reported that MBL protein inhibited infection by at least 50% for *C. trachomatis* C/TW-3/OT and E/UW-5/Cx at a concentration of 98 ng/ml and for *C. trachomatis* L2/434/Bu, *C. pneumoniae* AR-39 and *C. psittaci* 6BC at 6250 ng/ml. Those results indicate that MBL affects host immunity against chlamydia.

### 2.4 IL-6 and IL-6 receptor

IL-6 is a key pro-inflammatory cytokine that mediates several functions in inflammatory response through activated immune cells. IL-6 is produced most prominently by activated monocytes/macrophages, but also by T cells, endothelial cells, fibroblasts, mast cells and adipocytes. It has been proposed that about 25–30% of systemic IL-6 is produced by adipose tissue *in vivo* (Mohamed-Ali et al. 1997, Yudkin et al. 1999). The response of IL-6 in innate and adaptive immunity results in an acute phase response, differentiation and proliferation of T cells and growth of B cells.

IL-6 is an important activator of CRP (Heinrich et al. 1990). Slightly elevated serum CRP levels act as a marker of systemic inflammation and have been shown to increase the risk of cardiovascular diseases (Danesh et al. 1998, Danesh et al. 2000a). Elevated IL-6 plasma levels have also been associated with obesity, insulin resistance (Fried et al. 1998), type 2 diabetes (Pradhan et al. 2001) and CHD (Bennet et al. 2003, Jones et al. 2001a, Ridker et al. 2000b). Bennet et al. and Ridker et al. showed that men with elevated IL-6 or CRP levels had an increased risk for myocardial infarction.

The action of IL-6 in the target cells is initiated by formation of a complex with IL-6R. IL-6R is a type 1 membrane protein and it belongs to the cytokine receptor class 1 family. IL-6 first binds to the IL-6R α-receptor and then a signal-
transducing receptor component gp130 is recruited to the complex. Formation of the complex leads to activation of gp130-associated Janus kinase (JAK) tyrosine kinases and further to activation of transcription factors of the signal transducers and activators of transcription (STAT) family. The JAK/STAT intracellular signalling pathway finally results in an inflammatory response. (Heinrich et al. 2003, Heinrich et al. 1998.)

IL-6R is found in a membrane-bound form on hepatocytes and leucocyte (Hirata et al. 1989). Other cell types can be activated by a soluble form of IL-6R (sIL-6R) that lacks the transmembrane and cytoplasmic parts (Novick et al. 1989). sIL-6R binds to IL-6 and forms a complex with membrane-bound gp130. The complex mediates cellular response, thus acting as an IL-6R agonist (Jones et al. 2001b). The sIL-6R is produced by alternative splicing of the messenger RNA (mRNA) (Lust et al. 1992) or proteolytic cleavage of the membrane-bound receptor from a cleavage site at Gln357/Asp358 (Mullberg et al. 1994). Asp → Ala mutation in position 358 results in a reduced level of sIL-6R. The Asp358 allele is associated with increased sIL-6R levels (Galicia et al. 2004). A release of those two forms is differentially regulated in specific disease states. High levels of alternatively spliced sIL-6R was detected in serum of patients with adult T cell leukemia/human T cell leukemia virus-1-associated myelopathy, whereas high levels of sIL-6R formed by proteolytic cleavage are reported in AIDS patients (Honda et al. 1992, Horiuchi et al. 1998). (Jones et al. 2001b.)

2.4.1 IL-6 gene

The IL-6 gene is located at chromosome 7p21. Common polymorphisms have been described and the most studied is the promoter polymorphism -174 G/C (rs1800795), which has an influence on the transcription of the IL-6 gene and plasma levels of IL-6 (Fishman et al. 1998). The C allele has been found to be associated with lower levels of plasma IL-6 in healthy subjects and lower expression after LPS or IL-1 stimulation in HeLa cells (Fishman et al. 1998). On the contrary, the G/G genotype was associated with low IL-6 in abdominal aortic aneurysm patients (Jones et al. 2001a) and the C/C genotype was associated with higher production of IL-6 from LPS-stimulated mononuclear cells in neonates but not in adults (Kilpinen et al. 2001). It seems that regulation of IL-6 expression is cell-specific and may be dependent on age, gender, concomitant diseases, metabolic disorders and interactions between several polymorphisms (Terry et al. 2000).
The IL-6 gene has been suggested as a possible candidate gene for type 2 diabetes (Vozarova et al. 2003), systemic juvenile rheumatoid arthritis (Fishman et al. 1998) and cardiovascular diseases (Humphries et al. 2001). Humphries et al. reported an association between the C allele and higher systolic blood pressure, CRP level and risk of CHD in male patients. Georges et al. (2001) and Licastro et al. (2004) have reported an association between the C allele and myocardial infarction in male patients and Jenny et al. (2002) found the same in male and female patients. A lack of association has also been reported (Basso et al. 2002, Bennet et al. 2003, Lieb et al. 2004, Nauck et al. 2002, Sie et al. 2006). IL-6 messenger RNA has been shown to be expressed in human atherosclerotic lesions (Seino et al. 1994) and increased levels of IL-6 and IL-8 protein levels were found in atherosclerotic artery walls compared with those with normal intima (Rus et al. 1996). Those studies indicated a local production of IL-6 and a role of IL-6 in the atherogenetic process.

In recent Finnish studies, the -174G/C polymorphism was associated with risk factors and early markers of atherosclerosis (Hulkkonen et al. 2009, Riikola et al. 2009). The G allele genotype was significantly associated with higher HDL cholesterol and apolipoprotein A1 and lower systolic and diastolic blood pressure in young male subjects aged 24–39 years (Hulkkonen et al. 2009). In a middle-aged to elderly population (46–76 years), the G allele genotype was associated with higher serum total cholesterol and higher LDL cholesterol, lower plasma fasting glucose levels and lower BMI (Riikola et al. 2009). The IL-6 C allele has also been reported to be disadvantageous for longevity, as the G allele genotype was more prevalent in the survivors than in the non-survivors in a follow-up study of nonagenarians (Hurme et al. 2005).

Furthermore, the -174G/C polymorphism has been associated with a risk of upper respiratory tract infection and susceptibility to otitis media in children (Patel et al. 2006, Revai et al. 2009). IL-6, among other cytokines like IL-1β, IL-8, and TNFα, regulates the proliferation, chemotaxis and activation of inflammatory cells in the pathogenesis of inflammation in the respiratory tract.

### 2.4.2 IL-6R gene

The IL-6R gene is located at chromosomes 1q21. Several SNPs of the IL-6R gene have been identified; -183G/A (rs4845617) in the promoter region and 48892A/C (rs8192284) in exon 9 are the most studied (Kim et al. 2003). 48892A/C in the cleavage site has been associated with increased circulating levels of sIL6R
(Galicia et al. 2004) and obesity (Esteve et al. 2006, Wolford et al. 2003), metabolic syndrome (Esteve et al. 2006), diabetes (Hamid et al. 2004) and periodontitis (Galicia et al. 2006). In addition, 48892A/C was associated with elevated serum IL-6 (Qi et al. 2007, Reich et al. 2007) and CRP levels (Qi et al. 2009, Ridker et al. 2008). An increase in the IL-6/sIL-6R complex can enhance IL-6 signal transduction and activate cells that do not express the membrane-bound receptor and thus promote insulin resistance, obesity and diabetes. Promoter polymorphism -183G/A has been associated earlier with elevated BMI and obesity (Bustamante et al. 2007), but no association was found with periodontitis (Galicia et al. 2006).

2.5 Infection and inflammation

2.5.1 CRP – a marker of inflammation

CRP is an acute-phase protein synthesised by the liver in response to an inflammatory stimulus. A CRP serum concentration of 10 mg/l is considered the limit for a clinically significant inflammatory state and lower concentrations are regarded as indicating low-grade inflammation. Mean CRP levels in healthy European individuals range from 0.75 to 2.40 mg/l and increase slightly with age (Hutchinson et al. 2000). Variation in baseline CRP levels is affected by genetic variations, particularly in the CRP gene (Danik & Ridker 2007). Age, sex, BMI, smoking, socioeconomic factors, dietary factors and alcohol intake also have an influence on the CRP level (Eklund 2009). High-sensitivity (hs) CRP refers to measurement of serum CRP concentrations by using an immunoassay method that has a lower detection limit than a general laboratory test does. The test measures baseline CRP levels rather than response to an acute infection, and therefore it is used to detect CRP levels associated with low-grade systemic inflammation.

The release of CRP is mediated by IL-1, IL-6 and IL-17 (Heinrich et al. 1990). Also the production of IL-6 in subcutaneous adipose tissue regulates CRP concentration (Mohamed-Ali et al. 1997), and partially for this reason, increased baseline serum CRP levels are seen in obesity (Visser et al. 1999). CRP concentration has also been shown to decrease after weight loss (Eklund et al. 2006, Esposito et al. 2003, Heilbronn et al. 2001).

Low-grade inflammation is associated with many common inflammatory diseases and slightly elevated CRP levels are regarded as a risk for developing
insulin resistance (Festa et al. 2000, Yudkin et al. 1999), metabolic syndrome (Ridker et al. 2003), type 2 diabetes (Pradhan et al. 2001) and cardiovascular diseases (Danesh et al. 2000a, Ridker et al. 1997, Ridker et al. 2000a). Studies suggest that CRP may directly affect the artery wall, and CRP has been shown to be present in atherosclerotic plaque (Zhang et al. 1999). CRP also opsonises LDL for phagocytosis by macrophages (Zwaka et al. 2001).

2.5.2 Obesity – an inflammatory disease

Adipose tissue is composed of adipocytes, but it also includes fibroblasts, endothelial cells, monocytes and macrophages that constitute a stromal vascular fraction. Some of those cells are important in the immune system, and therefore adipose tissue plays a critical role in the immune response. Adipose tissue has a proinflammatory potential as it produces and expresses many cytokines and chemokines, including TNF-α, IL-6, IL-1β, IL-8 and IL-10. Furthermore, the macrophage accumulation is seen and expression of inflammatory cytokines is increased in obesity. (Cancello & Clement 2006, Desruisseaux et al. 2007, Hotamisligil et al. 1993, Mohamed-Ali et al. 2001.) Adipose tissue also produces and secretes adipokines like adiponectin, leptin and resistin, which regulate energy homeostasis and also play a role in inflammation and immune response (Desruisseaux et al. 2007). Approximately 30% of the circulating IL-6 is produced by adipose tissue (Mohamed-Ali et al. 1997). Synthesis of CRP is regulated by IL-6, and CRP has been associated with insulin resistance and obesity, as well (Visser et al. 1999, Yudkin et al. 1999). The increase in the expression of inflammatory molecules induces insulin resistance, increases lipolysis, increases leptin production and decreases adiponectin secretion by adipocytes (Desruisseaux et al. 2007, Xu et al. 2003). Low-grade inflammation of adipose tissue affects the pathogenesis of metabolic syndrome and obesity, and systemic consequences such as type 2 diabetes and cardiovascular diseases are seen (Pickup & Crook 1998, Ritchie & Connell 2007, Ross 1999).

Infections and obesity

In addition to genetic and environmental factors, obesity has been associated with infections. It is now known that infections and micro-organisms can cause adipocyte dysfunction and promote inflammatory reactions. Several associations between micro-organisms and obesity have been reported. In animals, canine
distemper virus, rous-associated virus-7 (RAV-7), scrapie agent, borna disease virus, avian adenovirus SMAM-1 and human adenovirus Ad-36 have been associated with obesity syndromes (Dhurandhar 2001). In humans, SMAM-1 and Ad-36 antibody-positive subjects have a higher BMI compared with antibody-negative subjects (Atkinson et al. 2005, Dhurandhar et al. 1997). In addition, Ad-36 has been shown to infect adipocytes and enhance differentiation of preadipocytes (Vangipuram et al. 2004). It has been postulated that Ad-36 has a direct effect on adipogenesis and/or the accumulation of lipid by adipocytes (Dhurandhar et al. 2000, Vangipuram et al. 2004). However, reversed causality has also been suggested. It has been found that overweight children are significantly more susceptible to acute respiratory tract infections than are normal-weight children (Jedrychowski et al. 1998), and one study suggested that obese individuals may have impaired lymphocyte response compared with non-obese subjects, leading to higher susceptibility to infections (Tanaka et al. 1993).

Recently, other micro-organisms, such as H. pylori and C. pneumoniae have been linked to an elevated BMI and other cardiovascular risk factors. First, Ekesbo et al. (2000) found that combined seropositivity for H. pylori and C. pneumoniae or either of them was associated with a high BMI and high fasting levels of insulin. Dart et al. (2002) also reported an association between C. pneumoniae seropositivity and cardiovascular risk factors, especially increased body weight. Karppinen et al. (2003) found that possible chronic C. pneumoniae infection, defined as persistent IgG or IgA antibodies in two serum samples taken at least one month apart, was associated with increased BMI levels in patients with sciatica. In a recent study (Lajunen et al. 2008a), elevated serum chlamydial LPS and C. pneumoniae IgG seropositivity, suggesting active frequent or chronic infection, were associated with an elevated BMI in patients with cardiovascular disease. In another recent study (Thjodleifsson et al. 2008), elevated BMI levels were more frequent in those with an elevated IgG antibody level for C. pneumoniae, as well as for H. pylori. On the contrary, other studies did not find any association between BMI and C. pneumoniae IgG seropositivity (Kaftan & Kaftan 2000, Koziolek et al. 2008). The interaction between C. pneumoniae and lipid metabolism has also been studied (Leinonen 2000, Leinonen & Saikku 1999). In Finnish reindeer herders, persistent C. pneumoniae antibodies over a period of three years were associated with lower concentrations of HDL cholesterol and higher concentrations of triglycerides (Laurila et al. 1997b) and, in addition, with obesity (BMI ≥ 28 kg/m²) (Leinonen & Saikku 1999).
Recently, it was shown that also *C. pneumoniae* is able to infect murine pre-adipocytes and adipocytes (Shi *et al.* 2008) as well as human adipocytes (Bouwman *et al.* 2008). In adipose tissue, *C. pneumoniae* promotes secretion of TNF-α and other inflammatory molecules, suppressing insulin signalling and adipogenesis (Shi *et al.* 2008).
3 Aims of the study

The aim of this work was to investigate if selected gene polymorphisms of innate immunity are associated with respiratory tract infections in young, healthy Finnish men during their military service. In addition, our aim was to study the hypothesis that chronic *C. pneumoniae* infection is linked with inflammation-related conditions. The specific aims were:

1. To define the serum MBL concentrations and *MBL2* genotypes in a young, healthy Finnish male population.
2. To study the association between both MBL levels and *MBL2* gene polymorphisms and respiratory tract infectious episodes during military service.
3. To investigate whether the MBL levels and *MBL2* gene polymorphisms are associated with the presence of *C. pneumoniae* antibodies as well as with seroconversions during military service.
4. To study if the selected polymorphism in the *IL-6* and *IL-6R* genes are connected with respiratory tract infectious episodes.
5. To study whether the *IL-6* gene polymorphism is associated with persistence of *C. pneumoniae* antibodies, a possible indicator of chronic infection.
6. To study the interaction between both persistent *C. pneumoniae* antibodies, a possible indicator of chronic infection, and slightly elevated CRP levels, a marker of low-grade inflammation, and BMI.
4 Materials and methods

4.1 Study subjects and specimens

The study population in total included 893 military recruits from the July 2004 and January 2005 intake groups for compulsory military service in Kajaani Garrison, Kainuu Brigade, in northern Finland. A description of the study population is shown in Table 4. All the men with medically diagnosed asthma were invited to participate. Randomly chosen men were invited after the asthmatic men had been excluded from the intake group. Twenty-five percent of all the men declined to participate and 3% were discharged because of health problems. All the men who agreed to participate in the study signed an informed consent form. Sera were obtained at the beginning and end of their service and paired sera, during each respiratory infectious episode diagnosed by a physician. A questionnaire was used at the beginning of their service to get information about factors affecting respiratory health. The study protocol was approved by the Medical Ethics Committee of the Kainuu Central Hospital.

Study I included only military recruits from the January 2005 intake group, and the statistical analyses of the infectious episodes were done in the group of men in the service for 180 days. Study III included men with IL-6 and IL-6R genotype data available from both intake groups in the service for 180 days. In studies II and IV, the study population consisted of the whole study population with limitation to available data on C. pneumoniae antibodies and BMI, respectively. The flow chart of the study populations is shown in Fig. 2.

Blood samples were collected and the leukocytes and sera were separated and stored at -70 °C or -20 °C, respectively, for later analysis.
Table 4. Description of the study populations (modified after Juvonen et al. (2008c))

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All (n = 893)</th>
<th>July 2004 (n = 420)</th>
<th>January 2005 (n = 473)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (n = 892), mean (SD)</td>
<td>19.6 (0.8)</td>
<td>19.6 (0.9)</td>
<td>19.6 (0.6)</td>
</tr>
<tr>
<td>Asthma, % (n)</td>
<td>25.6 (229)</td>
<td>28.1 (118)</td>
<td>23.5 (111)</td>
</tr>
<tr>
<td>Current smoker (n = 877), % (n)</td>
<td>43.1 (378)</td>
<td>38.9 (161)</td>
<td>46.9 (217)</td>
</tr>
<tr>
<td>Service time, % (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180 day</td>
<td>58.1 (518)</td>
<td>56.0 (235)</td>
<td>60.0 (283)</td>
</tr>
<tr>
<td>270 day</td>
<td>6.2 (55)</td>
<td>5.2 (22)</td>
<td>7.0 (33)</td>
</tr>
<tr>
<td>362 day</td>
<td>27.5 (245)</td>
<td>29.8 (125)</td>
<td>25.4 (120)</td>
</tr>
<tr>
<td>Study aborted</td>
<td>8.3 (74)</td>
<td>9.0 (38)</td>
<td>7.6 (36)</td>
</tr>
<tr>
<td>BMI (kg/m²) (n = 891), geometric mean (95% CI)</td>
<td>24.2 (23.9-24.4)</td>
<td>24.5 (24.1-24.8)</td>
<td>23.9 (23.6-24.3)</td>
</tr>
<tr>
<td>Education (n = 833), % (n)</td>
<td>91.0 (758)</td>
<td>90.2 (357)</td>
<td>91.8 (401)</td>
</tr>
</tbody>
</table>

1 Numbers of participants with data available shown, 2 Further education after comprehensive school

n, number; SD, standard deviation; BMI, body mass index; CI, confidence interval

Fig. 2. Flow chart of the study populations and number of individuals in each study.
4.1.1 Definitions

Asthma. Asthma was defined as previously diagnosed by a physician, according to data from the questionnaire and previous health and call-up examinations. The questionnaire at the beginning of military service included a question about the men’s own estimate of their medically diagnosed asthma (yes/no/don’t know). Data from previous examinations were used as confirmation.

Body mass index (BMI). The body height and weight of the conscripts were measured during the health examinations at the beginning of military service. BMI was calculated as weight in kilograms divided by the square of height in meters.

Smoking. Smoking was classified as smokers and non-smokers on the basis of the questionnaire at the beginning of military service. Smokers were current daily smokers. Non-smokers had never smoked or had stopped smoking or smoked only occasionally.

Education. The information on education was collected from the conscripts’ personal data forms and classified as comprehensive school or vocational education and upper secondary school.

4.1.2 Acute episodes of respiratory tract illness (I, III)

Infections (bacterial or viral) were defined by means of a combination of respiratory tract symptoms, physical findings and, if necessary, laboratory and radiological examinations. The conscripts with acute or acutely aggravated respiratory tract symptoms were checked by a nurse and examined by a physician, if necessary, in the military primary care clinic in the Kainuu Brigade. The episode was included in our database if a respiratory tract infection was diagnosed by the physician. Mild episodes not referred to a physician were not included in our analyses. The diagnosis of respiratory tract infection was based on symptoms and clinical findings. Consultations within two weeks of each other were considered as one episode. Symptoms, clinical findings and drug prescriptions were recorded. 90.7% of the episodes were diagnosed as an upper respiratory tract infection (common cold, sinusitis, pharyngitis and otitis), 6.6% as a lower respiratory tract infection (bronchitis and pneumonia) and 2.7% as both. Exacerbations of asthma were not included in this work. The statistical analysis of infectious episodes was done in the group of men in the service for 180 days, because the men with either 270- and 362-day service were partly trained in other
units in Finland, and therefore all of their infectious episodes may not have been included in our analysis. The men in 180-day service completed the follow-up.

4.2 DNA extraction (I, II, III)

The leukocytes were homogenised with 400 μl of homogenisation buffer (50 mM Tris-HCl, 10 mM NaCl, 50 mM EDTA, 1% SDS, pH 8.0) and 20 μl of Proteinase K (Sigma-aldrich co, Missouri, USA) three times at 4 m/s for 20 s. DNA was isolated from the entire homogenate with a Magna Pure LightCycler (LC) instrument (Roche Diagnostics GmbH, Mannheim, Germany) using a Large Volume Kit (Roche Diagnostics) by first following the DNALVBlood30_500 protocol and later the DNACVcells protocol. DNA was eluted with 200 μl of elution buffer. DNA concentrations were measured from the samples with a LightCycler instrument using a Quant-itTM PicoGreen dsDNA Assay Kit (Invitrogen-Molecular Probes, Eugene, USA). λ-DNA (100 μg/ml) was used as a standard at concentrations of 2 μg/ml, 0.2 μg/ml, 0.02 μg/ml and 0.002 μg/ml. The DNA was stored at −20 °C.

4.3 MBL2 genotyping (I, II)

A real-time PCR method for genotyping with the LightCycler Instrument (Roche Diagnostics GmbH) was set up according to a previously published method (Steffensen et al. 2003). The PCR was carried out using a LightCycler real-time PCR machine 1.2 and software version 4.05. Table 5 describes the MBL2 polymorphisms that were genotyped and Table 6 shows the names and sequences of the primers and probes that were used. MBLe forward (for) and reverse (rev) primers are for amplification of exon 1 and MBLp primers for the promoter region. The names of the detection (dete) and anchor (anch) probes indicate the variant alleles that the probes define. The probes were labelled with fluorescein (FL) and LightCycler Red 640 (LCRed640) or 705 (LCRed705) dyes. All the primers and probes were synthesised at Tib Molbiol Syntheselabor GmbH (Berlin, Germany).
Table 5. Description of the MBL2 polymorphism.

<table>
<thead>
<tr>
<th>SNP name</th>
<th>rs number</th>
<th>Gene region</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>H/L</td>
<td>rs11003125</td>
<td>-550</td>
<td>G/C</td>
</tr>
<tr>
<td>X/Y</td>
<td>rs7096206</td>
<td>-221</td>
<td>C/G</td>
</tr>
<tr>
<td>P/Q</td>
<td>rs7095891</td>
<td>5'UTR (+4)</td>
<td>C/T</td>
</tr>
<tr>
<td>A/D</td>
<td>rs5030737</td>
<td>Exon 1 codon 52</td>
<td>C/T</td>
</tr>
<tr>
<td>A/B</td>
<td>rs1800450</td>
<td>Exon 1 codon 54</td>
<td>G/A</td>
</tr>
<tr>
<td>A/C</td>
<td>rs1800451</td>
<td>Exon 1 codon 57</td>
<td>G/A</td>
</tr>
</tbody>
</table>

Table 6. Primers and probes for MBL2 gene genotyping.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers</td>
<td></td>
</tr>
<tr>
<td>MBlE for</td>
<td>CATCAACGGCTTCCCCAGGC</td>
</tr>
<tr>
<td>MBlE rev</td>
<td>TGGGCTGGCAAGACAATTTAG</td>
</tr>
<tr>
<td>MBlp for</td>
<td>CCTGCCAGAAAGTAGAGAGG</td>
</tr>
<tr>
<td>MBlp rev</td>
<td>CCTCACCTTGGTGTGAGAAA</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Probes</td>
<td></td>
</tr>
<tr>
<td>MBlE anch</td>
<td>CAGCCCAACAGTGACCTGGTCCCTCCCT-FL</td>
</tr>
<tr>
<td>MBlE dete</td>
<td>LCRed640-TTCTTCTCGTGCACGGCA-PH</td>
</tr>
<tr>
<td>MBl H/L dete</td>
<td>TTTTAGACAGGCTTGGCTGCTG-FL</td>
</tr>
<tr>
<td>MBl H/L anch</td>
<td>LCRed640-AGCATTTTCTCTGGAAATTTTCTACTACGTTGG-PH</td>
</tr>
<tr>
<td>MBl P/Q dete</td>
<td>CAGAGGCCATGTCGCTGAA-FL</td>
</tr>
<tr>
<td>MBl P/Q anch</td>
<td>LCRed640-ATGTGATCATTTACATGAGATGGAACCTCTGAG-PH</td>
</tr>
<tr>
<td>MBl X/Y dete</td>
<td>TCTCACCCTGACCGAAGCAT-FL</td>
</tr>
<tr>
<td>MBl X/Y anch</td>
<td>LCRed705-TTCTATATGCTTCCAGGCAACCGCCA-PH</td>
</tr>
</tbody>
</table>

MBlE, mannose-binding lectin exon 1 primer; MBlp, MBL promoter primer; for, forward primer; rev, reverse primer; FL, fluorescein label; LCRed640/705, LightCycler Red 640/705 dye; PH, phosphate; dete, detection probe; anch, anchor probe

The reaction (20 μl) for exon 1 genotyping contained approximately 2.9-436 ng (2 μl of each sample) of DNA, 0.5 μM of both primers, 0.4 μM of the anchor probe, 0.2 μM of the detection probe, 3.5 mM MgCl2 and 2 μl of 10x LightCycler DNA Master Hybridization Probes reagent (Roche Diagnostics GmbH). The PCR program consisted of initial denaturation at 95 °C for 10 minutes, followed by amplification for 45 cycles of denaturation at 95 °C for 0 s (20 °C/s), annealing at 60 °C for 10 s (3 °C/s) and extension at 72 °C for 9 s (20 °C/s). A melting curve analysis was performed for 1 cycle at 95 °C for 0 s (20 °C/s) and 50 °C for 3 min (20 °C/s), and finally the temperature was raised to 85 °C for 0 s at a rate of 0.2 °C/s. Fluorescence was measured continuously during the temperature rise.
The PCR reaction mix for H/L genotyping was the same as for exon 1 genotyping. The PCR protocol consisted of initial denaturation at 95 °C for 10 minutes, amplification for 45 cycles of denaturation at 95 °C for 0 s (20 °C/s), annealing at 57 °C for 10 s (3 °C/s) and extension at 72 °C for 20 s (20 °C/s). The melting curve analysis was performed for 1 cycle at 50 °C for 0 s at a rate of 10 °C/s, at 45 °C for 3 min ( 20 °C/s), with final heating to 75 °C for 0 s (0.2 °C/s).

X/Y and P/Q genotyping was performed with dual colour under the same conditions as for H/L genotyping. As an exception to exon 1, the reaction mix contained 0.5 μM of both primers, 0.2 μM of each of the detection and anchor probes and 4 mM MgCl₂. The P/Q variant was detected with the LC-Red 640 channel (F2) and the X/Y variant was detected with the LC-Red 705 channel (F3). Both the X/Y and P/Q variants could be detected by using colour compensation (Colour Compensation set Roche Diagnostics), which corrects signal overflow between channels 2 and 3.

To verify the exon 1 genotypes, samples representing different genotypes were sequenced by Macrogen Inc. (Seoul, South Korea). In addition, known controls were analysed together with the samples in each genotyping assay.

4.4 Measurement of serum MBL concentration (I, II)

The MBL concentrations of the serum samples obtained at the beginning of military service were measured using a Human MBL (Lectin assay) enzyme-linked immunosorbent assay (ELISA) test kit (Hycult Biotechnology, Uden, The Netherlands) according to the manufacturer’s instructions. Absorption at 450 nm was measured with a Multiscan Ascent plate reader (Thermo Electron Corporation, Waltham, USA). A standard curve was created, and the concentrations were defined using Ascent software version 2.6 (Thermo Electron Corporation).

4.5 IL6 and IL6R genotyping (III)

SNPs in the IL-6R and IL-6 genes were selected after evaluation of the literature, The National Center for Biotechnology (NCBI) and HapMap tag SNP data for the genes. The SNPs were chosen on the basis of the following criteria: 1) minor allele frequency (MAF) ≥ 0.30 (in order to achieve proper group sizes), 2) functionality (coding SNPs, possible function reported in literature), 3) tag SNPs
that captured other SNPs. The SNPs included one coding SNP and four non-coding SNPs in the *IL-6R* gene and two non-coding SNPs in the *IL-6* gene (Table 13 and Table 14 in section 5.5). Genotyping was performed by MALDI-TOF mass spectrometry (Matrix-Assisted Laser Desorption-Ionisation-Time of Flight; Sequenom GmbH, San Diego, CA, USA) in a Mutation Analysis Facility, Karolinska Institute, Stockholm, Sweden. Validation included positive and negative controls and concordance genotyping. The overall genotyping success rate was > 95% for each SNP, and all the SNPs were in Hardy-Weinberg equilibrium. Three individuals were excluded due to complete genotyping failure.

### 4.6 Microimmunofluorescence test for measurement of *C. pneumoniae* antibodies (II, IV)

A MIF test was used to measure *C. pneumoniae* IgG, IgA and IgM antibody levels in the serum samples from the military recruits according to a previously published protocol (Paldanius *et al.* 2003, Wang 2000). Using Finnish *C. pneumoniae* strain Kajaani 6 (K6) elementary bodies as an antigen, serial twofold dilutions of the serum samples were done to detect antibodies. Positive serum controls with known titres were included in each series. The seropositivity cut-off value for IgG, IgA and IgM antibodies were ≥ 32, ≥ 10 and ≥ 8, respectively. In study II, the presence of *C. pneumoniae* IgM in any serum during service or at departure and/or a ≥ fourfold antibody rise in IgG or IgA antibodies between any paired serum samples was considered an indication of clinical or subclinical *C. pneumoniae* infection.

In study IV and in some unpublished results, the presence of *C. pneumoniae* IgG antibodies in both arrival and departure serum samples during 180-, 270-, or 362-day service was considered a persistence of antibodies and a possible indication of chronic *C. pneumoniae* infection. A fourfold or greater rise in IgG antibodies between the arrival and departure samples was considered a seroconversion and a possible indication of clinical or subclinical *C. pneumoniae* infection.

### 4.7 High-sensitivity C-reactive protein (hsCRP) (IV)

The hsCRP levels were determined with an Immunoenzymometric Assay (IEMA) test (Medix Biochemica, Kauniainen, Finland) from the blood samples according to the manufacturer’s instructions (Taponen *et al.* 2004). The assay range was
from 0.3 to 30 mg/l, and the sensitivity of the test was 0.08 mg/l. The lowest hsCRP level of the highest quartile was used as a cut-off value for elevated hsCRP levels. To limit the possible increasing influence of infections on the CRP level, only conscripts with CRP < 10 mg/l were included in the CRP analysis. A persistent CRP level was defined as a CRP level over the upper quartile in the arrival and departure samples during service.

### 4.8 Statistical analysis

In describing the respiratory tract infections and MBL measures of the study population and in studies I and II, the median MBL levels between different groups were analysed with a Mann-Whitney U test and a Kruskal-Wallis test. A Pearson Chi-squared test, or Fisher’s exact test, if necessary, was used to evaluate the statistical significance of the association between categorised variables. Due to the multiple comparisons (I), the p values were corrected using the Benjamini-Hochberg procedure ($p_{corrected}$), which took into consideration the number of comparisons (Benjamini & Hochberg 1995). Logistic regression analysis was used for multivariate analysis. MBL2 haplotypes were reconstructed with the statistical PHASE programme described by Stephens and co-workers (Stephens et al. 2001). The current version 2.1 (code by Stephens 2004), with some extensions to the original method, was used.

Pearson Chi-squared test was used in the analysis of IL-6 and IL-6R polymorphisms (study III and some unpublished results). Associations were analysed for each polymorphism using additive, genotypic, recessive and dominant models. To control multiple testing (III), empirical p values were obtained using 5000 permutations for each SNP. Logistic regression for categorised variables and Poisson regression for continuous variables were used for multivariate analyses. Haploview 4.1. software (Barrett et al. 2005) was used to analyse linkage disequilibrium (LD) between the SNPs and to form and analyse haplotypes.

In study IV, an independent T test was used for continuous variables and Pearson Chi-squared test was used for categorised variables to test the significance between BMI groups. For continuous variables that did not follow normal distribution, a logarithmic transformation was done. Logistic regression and analysis of variance were used for multivariate analyses.

The statistical analyses were done using SPSS for Windows version 13.0, 15.0 and 18.0 (SPSS Inc. Chicago, Illinois, USA), and the analysis of IL-6 and IL-
polymorphisms was done with PLINK for MS DOS v1.02. (Purcell et al. 2007) (URL: http://pngu.mgh.harvard.edu/purcell/plink/).

In addition, post-study power calculations were performed using Genetic Power Calculator (Purcell et al. 2003) and Stata 5.0 softwares (Stata Corporation Inc., College Station, Texas, USA). A genetic power calculation of study I indicated that when the MAFs of MBL2 polymorphisms were 0.2, odds ratio (OR) = 1.5 and 20% occurrence of at least two respiratory tract infectious episodes was assumed in the general military population, this study had 15% power for the dominant genetic model at a 5% significance level. For study II, 30% power was achieved for the dominant model when 20% occurrence of C. pneumoniae seroconversion was assumed. For IL-6 and IL-6R analysis (III), this study had 75%, 83% and 62% power for the genotypic, additive and recessive genetic model at a 5% significance level when the MAFs were 0.3, OR = 1.5 and 50% occurrence of at least one respiratory infectious episode was assumed. The study IV had 81% power at a 5% significance level to detect 10% difference in C. pneumoniae seroprevalence between the normal-weight and overweight conscripts when 40% prevalence was assumed in the normal-weight group.
5 Results

5.1 Respiratory tract infections in the study population

5.1.1 Respiratory tract infectious episodes

Five hundred eighteen men in 180-day service experienced a total of 409 respiratory tract infectious episodes during their 180-day military service, 145 among those in the July 2004 intake group and 264 among those in the January 2005 intake group. The infectious episodes were categorised for the association analyses as follows: zero versus at least one episode ($\geq 1$), zero to one episode versus at least two episodes ($\geq 2$) and zero to two episodes versus frequent episodes ($\geq 3$). The frequencies of episodes in the men during 180-day service are shown in Table 7. Two hundred sixty-six men had at least one infectious episode and 252 men had no episodes during their service.

The conscripts with asthma had significantly more respiratory tract infections during their 180-day service. At least two episodes were diagnosed in 29.1% of men with asthma and in 16.4% of men without asthma ($p = 0.002$). Infections were also more common in the January 2005 than in the July 2004 intake group (Table 7).

Table 7. Respiratory tract infectious episodes in men during 180-day military service

<table>
<thead>
<tr>
<th>Infectious episode classification</th>
<th>All, % (n)</th>
<th>July 2004, % (n)</th>
<th>January 2005, % (n)</th>
<th>p value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\geq 1$ episode</td>
<td>51.4 (266)</td>
<td>41.3 (97)</td>
<td>59.7 (169)</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>$\geq 2$ episodes</td>
<td>19.5 (101)</td>
<td>15.7 (37)</td>
<td>22.6 (64)</td>
<td>0.049</td>
</tr>
<tr>
<td>$\geq 3$ episodes</td>
<td>6.4 (33)</td>
<td>4.3 (10)</td>
<td>8.1 (23)</td>
<td>0.072</td>
</tr>
</tbody>
</table>

$^1$ Pearson Chi-squared test between intake groups

5.1.2 C. pneumoniae antibodies

C. pneumoniae antibodies were studied in the men with serological data available during 180-, 270- and 362-day service. The serological results and the differences between the two intake groups are presented in Table 8. IgA seropositivity ($\geq 40$) at departure ($p = 0.007$), IgG seropositivity on arrival ($\geq 32$: $p = 0.003$ and $\geq 128$: $p = 0.025$) and persistence of IgG antibodies ($p = 0.003$) were more common in conscripts with asthma than in those without.
Table 8. Description of C. pneumoniae antibodies in the study population (modified after Paldanius et al. (2007)).

<table>
<thead>
<tr>
<th>C. pneumoniae antibodies</th>
<th>All, % (n)</th>
<th>July 2004, % (n)</th>
<th>January 2005, % (n)</th>
<th>p value1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 893</td>
<td>n = 420</td>
<td>n = 473</td>
<td></td>
</tr>
<tr>
<td>IgG ≥ 32 on arrival</td>
<td>45.9 (408)</td>
<td>54.3 (227)</td>
<td>38.4 (181)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IgG ≥ 32 at departure</td>
<td>40.9 (325)</td>
<td>34.7 (131)</td>
<td>46.4 (194)</td>
<td>0.001</td>
</tr>
<tr>
<td>IgG ≥ 128 on arrival</td>
<td>12.7 (113)</td>
<td>18.2 (76)</td>
<td>7.9 (37)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IgG ≥ 128 at departure</td>
<td>8.1 (64)</td>
<td>6.9 (26)</td>
<td>9.1 (38)</td>
<td>0.256</td>
</tr>
<tr>
<td>IgA ≥ 40 on arrival</td>
<td>8.1 (72)</td>
<td>6.7 (28)</td>
<td>9.3 (44)</td>
<td>0.152</td>
</tr>
<tr>
<td>IgA ≥ 40 at departure</td>
<td>7.0 (56)</td>
<td>8.5 (32)</td>
<td>5.7 (24)</td>
<td>0.131</td>
</tr>
<tr>
<td>IgG ≥ 128 and/or IgA ≥ 40 on arrival</td>
<td>16 (144)</td>
<td>15.3 (64)</td>
<td>17.1 (80)</td>
<td>0.472</td>
</tr>
<tr>
<td>(n = 887)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG persistence2</td>
<td>29.5 (234)</td>
<td>29.3 (110)</td>
<td>29.7 (124)</td>
<td>0.899</td>
</tr>
<tr>
<td>IgA persistence2</td>
<td>11.0 (87)</td>
<td>9.9 (37)</td>
<td>12.0 (50)</td>
<td>0.346</td>
</tr>
<tr>
<td>IgG seroconversion2</td>
<td>12.8 (102)</td>
<td>6.4 (24)</td>
<td>18.7 (78)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IgA seroconversion2</td>
<td>7.7 (61)</td>
<td>8.5 (32)</td>
<td>6.9 (29)</td>
<td>0.406</td>
</tr>
<tr>
<td>IgG/IgA seroconversion or IgM ≥ 10a</td>
<td>20.5 (163)</td>
<td>14.3 (54)</td>
<td>26.1 (109)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(n = 795)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Pearson Chi-squared test between intake groups, 2 Antibody persistence or seroconversion in arrival and departure sera, a Seroconversion between arrival, acute and departure sera or IgM ≥ 10 in the acute or departure sera

5.2 Serum MBL concentrations and MBL2 genotypes of the study population

The median (interquartile range) MBL concentration was 1156.6 ng/ml (431.9–1981.3 ng/ml) in the whole study population, 1219.1 ng/ml (411.7–1912.2 ng/ml) in the July 2004 intake group and 1087.5 ng/ml (454.8–2068.5 ng/ml) in the January 2005 intake group. The levels ranged from 0 to 7342.0 ng/ml. The difference between intake groups was not significant (p = 0.661). Neither was there any difference in the median MBL levels between those with asthma and those without (p = 0.927).

Table 9 presents the genotype frequencies of the MBL2 gene and Fig. 3 the MBL concentrations of the different genotypes in the whole study population. The MBL2 genotype and MBL concentration distributions of the January 2005 intake group are shown in study I, and its Table 2 and Fig. 1. There was no difference in the genotype distribution between those with asthma and those without. All the polymorphisms were in Hardy-Weinberg equilibrium. The H/H genotype was connected to high, H/L to intermediate and L/L to low MBL levels (p < 0.001).
All the X/X, X/Y and Y/Y genotypes were associated with intermediate MBL levels ($p = 0.136$). The P/P genotype was associated with intermediate, P/Q with high and Q/Q with very high concentrations ($p < 0.001$). The wild-type \textit{MBL2} exon 1 genotype A/A was associated with high MBL levels, heterozygous variant allele genotypes A/D, A/B and A/C with low levels and homozygous variant genotypes B/C, B/B, B/D and D/D with almost undetectable MBL levels ($p < 0.001$). The structural genotypes were categorised as A/A, A/O and O/O genotype groups, where O stands for any of the exon 1 variant alleles B, C or D. A significant difference in MBL levels was seen between these genotype groups ($p < 0.001$) (Fig. 3).

**Table 9. Frequencies of the \textit{MBL2} genotypes in the total study population.**

<table>
<thead>
<tr>
<th>MBL2 genotype</th>
<th>Frequency, % (n)</th>
<th>Homozygote 1</th>
<th>Heterozygote</th>
<th>Homozygote 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>-550 H/L (n = 885)</td>
<td>18.6 (165)</td>
<td>48.4 (428)</td>
<td>33.0 (292)</td>
<td></td>
</tr>
<tr>
<td>-221 X/Y (n = 886)</td>
<td>4.2 (37)</td>
<td>31.7 (281)</td>
<td>64.1 (568)</td>
<td></td>
</tr>
<tr>
<td>+4 P/Q (n = 887)</td>
<td>68.8 (610)</td>
<td>27.6 (245)</td>
<td>3.6 (32)</td>
<td></td>
</tr>
<tr>
<td>Exon 1 A/O (n = 887)</td>
<td>64.3 (571)</td>
<td>30.9 (274)</td>
<td>4.8 (43)</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Numbers of participants with data available shown. $^2$O stands for any of the exon 1 variant alleles B, C or D. $^a$Specific frequencies (%): A/D = 7.5, A/B = 21.4 and A/C = 1.9, $^b$B/C = 0.7, B/B = 1.7, B/D = 2.3 and D/D = 0.2.
Fig. 3. MBL concentrations of the different MBL2 genotypes in the whole study population (n = 893). In the exon 1 genotype groups, A refers to the wild-type allele and O refers to any of the exon 1 variant alleles B, C or D. January 2005 intake group is described in study I, Fig. 1.
5.3 The association between MBL and respiratory tract infections (I)

The MBL levels were categorised into two groups using a median MBL concentration of 1087.5 ng/ml as the limit. Significantly more conscripts with MBL concentrations under the median MBL level had at least two infectious episodes during their service (30% vs. 16%, respectively; \( p = 0.003, p_{\text{corrected}} = 0.011 \)) compared with those with over-median MBL levels. In addition, an MBL level below the median concentration proved to be a significant risk factor for infections: OR 2.5 (95% CI 1.4 – 4.5) when adjusted for asthma status.

Since there was no difference between the genotype distributions of those with asthma and those without, the association between infections and genotypes could be considered in both sample types together (Table 10). The association between infections and exon 1 genotypes was analysed for the high-MBL-producing wild-type genotype (A/A) and the low-MBL-producing combined heterozygous and homozygous variant allele genotype groups (A/O + O/O). The conscripts with the A/O or O/O genotype had a borderline significant risk of infections when adjusted for asthma status. In analysing the dependence between the MBL concentration groups, < 1087.5 ng/ml and > 1087.5 ng/ml, and the MBL2 structural genotype groups, A/A and A/O + O/O, we found that as many as 94% of those with the A/O or O/O genotype and 26% of those with the A/A genotype had MBL levels below the median (\( p < 0.001 \)). In addition, the Y/Y genotype was significantly more frequent in those with at least two infections compared with the X/X or X/Y genotype. To study the combined effect of the high-risk SNPs, Y/Y genotype and O allele, the infection episodes in the genotype groups YA/YO + YO/YO and XA/XA + XA/YA were analysed. The YA/YA and XA/YO groups were omitted from this analysis. The difference between these groups was significant (\( p = 0.025 \)).
Table 10. Association between MBL2 genotypes and respiratory tract infections (I).

<table>
<thead>
<tr>
<th>MBL2 genotype</th>
<th>Subject with ≥ 2 infections, % (proportion)</th>
<th>p₁</th>
<th>p_corrected ²</th>
<th>Adjusted OR (95% CI)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>-550</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H/H + H/L</td>
<td>24 (43/181)</td>
<td>0.659</td>
<td>0.769</td>
<td>1.3 (0.7-2.5)</td>
</tr>
<tr>
<td>L/L</td>
<td>21 (21/98)</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>-221</td>
<td></td>
<td>0.013</td>
<td>0.025</td>
<td>1.0</td>
</tr>
<tr>
<td>X/X + X/Y</td>
<td>15 (16/107)</td>
<td></td>
<td></td>
<td>2.3 (1.2-4.4)</td>
</tr>
<tr>
<td>Y/Y</td>
<td>28 (48/173)</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>+4</td>
<td></td>
<td>&gt; 0.99*</td>
<td>&gt; 0.99</td>
<td>1.0</td>
</tr>
<tr>
<td>P/P + P/Q</td>
<td>23 (62/269)</td>
<td></td>
<td></td>
<td>2.3 (1.2-4.4)</td>
</tr>
<tr>
<td>Q/Q</td>
<td>17 (2/12)</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Exon 1</td>
<td></td>
<td>0.190</td>
<td>0.266</td>
<td>1.0</td>
</tr>
<tr>
<td>A/A</td>
<td>21 (40/195)</td>
<td></td>
<td></td>
<td>1.7 (0.9-3.1)</td>
</tr>
<tr>
<td>A/O + O/O</td>
<td>28 (24/87)</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Haplotype</td>
<td></td>
<td>0.014</td>
<td>0.025</td>
<td>1.0</td>
</tr>
<tr>
<td>XA/XA + XA/YA</td>
<td>14 (12/86)</td>
<td></td>
<td></td>
<td>3.4 (1.4-7.9)</td>
</tr>
<tr>
<td>YA/YO + YO/YO</td>
<td>30 (20/66)</td>
<td></td>
<td></td>
<td>&gt; 0.99*</td>
</tr>
</tbody>
</table>

¹ Pearson Chi-squared test (if not otherwise mentioned), ² Benjamini-Hochberg procedure, ³ Odds ratio (95% confidence interval), adjusted for asthma status, ⁴ Fisher’s exact test

5.4 The association between MBL and C. pneumoniae infection (II)

5.4.1 MBL and C. pneumoniae seroconversion

The presence of IgM antibodies (seropositivity on arrival was not included) and IgG and IgA seroconversions between arrival, acute and departure sera during 180-, 270- and 362-day service were all considered a marker of being exposed to C. pneumoniae during service. MBL levels were categorised into two groups, using a median MBL concentration of 1151.6 ng/ml as a cut-off. MBL levels under the median concentration were significantly associated with IgG or IgA seroconversions or the presence of IgM antibodies during service, compared with over-median MBL levels (Table 11). In addition, the P/P genotype was more frequent than the P/Q or Q/Q genotype with those who were considered to have been exposed to C. pneumoniae during service (Table 11). When the below-median MBL level and the P/P genotype were combined and the presence of zero risk factors was compared with one and two factors, a significant linear trend was detected (13% vs. 19% vs. 26%, respectively; p = 0.002). The risk also grew...
when only one factor was compared with two factors (OR; 95% CI: 1.5; 0.9–2.7 and 2.1; 1.2–3.7, respectively).

Table 11. Association between both MBL levels and MBL2 genotypes and C. pneumoniae seroconversion during 180-, 270- and 362-day service.

<table>
<thead>
<tr>
<th>MBL level</th>
<th>Percent (n)</th>
<th>P value2</th>
<th>OR (95% CI)3</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; Md4</td>
<td>24 (95/397)</td>
<td>0.018</td>
<td>1.5 (1.1-2.1)</td>
</tr>
<tr>
<td>≥ Md</td>
<td>17 (68/398)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>550</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H/H</td>
<td>17 (26/149)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>H/L + L/L</td>
<td>21 (137/640)</td>
<td>0.283</td>
<td>1</td>
</tr>
<tr>
<td>221</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X/X + X/Y</td>
<td>22 (63/282)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Y/Y</td>
<td>20 (99/508)</td>
<td>0.341</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/P</td>
<td>23 (122/541)</td>
<td></td>
<td>1.5 (1.0-2.2)</td>
</tr>
<tr>
<td>P/Q + Q/Q</td>
<td>16 (40/250)</td>
<td>0.034</td>
<td>1</td>
</tr>
</tbody>
</table>

Exon 1
| A/A       | 21 (108/514)|          |             |
| A/O + Q/O | 20 (55/277) | 0.701    |             |

1 Seroconversions between arrival, acute and departure sera. IgM seropositivity in the acute or departure sera, 2 Pearson Chi-squared test, 3 Odds ratio (95% confidence interval), adjusted for asthma status and intake group, 4 Median MBL concentration was 1151.6 ng/ml.

5.4.2 MBL and C. pneumoniae seropositivity

The Y/Y genotype and the exon 1 variant allele genotypes (A/O and O/O) were significantly associated with the presence of elevated IgG and/or IgA antibodies when adjusted for asthma status and intake group. Furthermore, a borderline significant association was detected between the antibodies and the H/H genotype (Table 12).
Table 12. Association between both MBL levels and MBL2 genotypes and C. pneumoniae antibodies in military recruits on arrival.

<table>
<thead>
<tr>
<th>MBL level</th>
<th>Percent (n)</th>
<th>p value&lt;sup&gt;1&lt;/sup&gt;</th>
<th>OR (95% CI)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; Md&lt;sup&gt;3&lt;/sup&gt;</td>
<td>18 (74/444)</td>
<td>0.727</td>
<td>1.5 (0.9-2.3)</td>
</tr>
<tr>
<td>≥ Md</td>
<td>16 (70/443)</td>
<td>0.145</td>
<td>1.6 (1.1-2.3)</td>
</tr>
</tbody>
</table>

Table 13. Description of polymorphisms in the IL-6 gene.

<table>
<thead>
<tr>
<th>SNP name</th>
<th>rs number</th>
<th>Gene region</th>
<th>Alleles (major/minor)</th>
<th>MAF&lt;sup&gt;1&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6A</td>
<td>rs1800795</td>
<td>5'UTR (-174)</td>
<td>C/G</td>
<td>48.7</td>
</tr>
<tr>
<td>IL6B</td>
<td>rs1474347</td>
<td>Intron 2</td>
<td>C/A</td>
<td>48.8</td>
</tr>
</tbody>
</table>

<sup>1</sup>Minor allele frequency

5.5 IL-6 and IL-6R gene polymorphisms (III)

The description and allele frequencies of IL-6 and IL-6R gene polymorphisms in the 511 men in the service for 180 days are shown in Tables 13 and 14, respectively. In the IL-6 gene, IL6A and IL6B (r<sup>2</sup> = 0.99) (Fig. 4), and in the IL-6R gene, IL6R1 and IL6R2 (r<sup>2</sup> = 0.93) and IL6R4 and IL6R5 (r<sup>2</sup> = 0.47) (Fig. 5) were in a strong LD (D’ > 0.9).

There was no association between the IL-6 and IL-6R polymorphisms and asthma status, smoking or BMI.
Table 14. Description of polymorphisms in the IL-6R gene.

<table>
<thead>
<tr>
<th>SNP name</th>
<th>rs number</th>
<th>Gene region</th>
<th>Alleles (major/minor)</th>
<th>MAF(^1) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6R1</td>
<td>rs4845617</td>
<td>5'UTR (-183)</td>
<td>G/A</td>
<td>41.3</td>
</tr>
<tr>
<td>IL6R2</td>
<td>rs6427641</td>
<td>Intron 1</td>
<td>A/G</td>
<td>44.7</td>
</tr>
<tr>
<td>IL6R3</td>
<td>rs4601580</td>
<td>Intron 1</td>
<td>T/A</td>
<td>45.0</td>
</tr>
<tr>
<td>IL6R4</td>
<td>rs4845371</td>
<td>Intron 5</td>
<td>T/C</td>
<td>47.8</td>
</tr>
<tr>
<td>IL6R5</td>
<td>rs8192284</td>
<td>Exon 9 (48892)</td>
<td>A/C</td>
<td>30.9</td>
</tr>
</tbody>
</table>

\(^1\)Minor allele frequency

Fig. 4. LD map of the markers genotyped in the IL-6 gene. The dark square represent high LD. The location of polymorphisms is shown.
Fig. 5. LD map of the markers genotyped in the IL-6R gene. The dark squares represent high LD and the light squares represent low LD. The location of polymorphisms is shown.

### 5.5.1 The association between IL-6 and IL-6R polymorphisms and respiratory tract infections (III)

The association between the IL-6 and IL-6R genotypes and infectious episodes during military service was studied in two categorisations: zero versus at least one episode (≥1), and zero to two episodes versus frequent episodes (≥3). Homozygous genotypes A/A of IL6R1, G/G of IL6R2 and A/A of IL6R3 were associated with infectious episodes during service. Some of these associations also remained statistically significant or approached statistical significance after a permutation procedure. The other two polymorphisms in the IL-6R gene and the polymorphisms in the IL-6 gene were not associated with infections (study III, Table 2).
Six haplotypes comprising SNPs IL6R1 from LD block 1, IL6R3 between the blocks and IL6R5 from block 2 were detected with a frequency of over 5% of altogether eight possible haplotypes (study III, Table 3). GTC seemed to be a protective haplotype and AAC a risk haplotype, but the associations did not remain significant after permutation.

To further analyse the associations between the polymorphisms and infectious episodes, additive, dominant and recessive models (for the minor allele) as well as multivariate analyses were performed (Table 15). Under the additive and recessive models, the A and G alleles of IL6R1 and IL6R2, respectively, proved to be significant risk factors for infectious episodes. In addition, under the additive and recessive models, the A allele of IL6R3 was a risk factor for frequent infections (Table 15). According to the genotype frequencies between the cases and controls and the significance of the statistical tests, the recessive model seemed to be the best model for describing the association between polymorphisms and infections. Thus, the homozygous minor allele genotypes of the IL6R1, IL6R2 and IL6R3 polymorphisms were risk factors for respiratory tract infections.

A general linear model with a Poisson regression was fit for analysing the association between IL6R1, IL6R2, and IL6R3 and the number of infectious episodes (study III, Table 5). The results suggested that conscripts with the IL6R1 A/A genotype had a 71% greater risk of having infectious episodes during military service (OR 1.71, 95% CI 1.34–2.19). The suggested risks for conscripts with the IL6R2 G/G and IL6R3 A/A genotypes were 65% (OR 1.65, 95% CI 1.22–2.24) and 22% (OR 1.22, 95% CI 0.97–1.53), respectively.

A combination of the IL6R1 A/A and/or IL6A G/G genotypes in relation to infection episodes was studied (study III, Table 6). There was a significant trend for individuals who were carriers of both genotypes to have more infectious episodes than those who were carriers of only one of the genotypes or neither of the genotypes.
Table 15. **IL-6R gene polymorphisms and the risk of having respiratory tract infectious episodes during military service.**

<table>
<thead>
<tr>
<th></th>
<th>SNP</th>
<th>≥ 1 infection cases/controls, %</th>
<th>p</th>
<th>OR (95% CI)</th>
<th>≥ 3 infections cases/controls, %</th>
<th>p</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL6R1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele G</td>
<td>54.7/62.8</td>
<td>1</td>
<td></td>
<td>44.2/59.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele A</td>
<td>45.3/37.2</td>
<td>0.012 (0.063)</td>
<td>1.48 (1.12-1.97)</td>
<td>55.8/40.5</td>
<td>0.030 (0.146)</td>
<td>1.92 (1.06-3.48)</td>
</tr>
<tr>
<td></td>
<td>R model</td>
<td>&lt; 0.001 (0.005)</td>
<td>2.63 (1.52-4.54)</td>
<td></td>
<td>0.001 (0.011)</td>
<td>3.76 (1.60-8.85)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL6R2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele A</td>
<td>51.7/59.3</td>
<td>1</td>
<td></td>
<td>42.2/56.2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele G</td>
<td>48.3/40.7</td>
<td>0.015 (0.083)</td>
<td>1.40 (1.07-1.82)</td>
<td>57.8/43.8</td>
<td>0.029 (0.139)</td>
<td>1.77 (1.03-3.03)</td>
</tr>
<tr>
<td></td>
<td>R model</td>
<td>0.003 (0.015)</td>
<td>2.08 (1.29-3.35)</td>
<td></td>
<td>0.006 (0.032)</td>
<td>2.77 (1.28-6.00)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL6R3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele T</td>
<td>52.5/57.6</td>
<td>1</td>
<td></td>
<td>40.6/56.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allele A</td>
<td>47.5/42.4</td>
<td>0.120 (0.444)</td>
<td></td>
<td>59.4/44.0</td>
<td>0.017 (0.087)</td>
<td>1.81 (1.06-3.10)</td>
</tr>
<tr>
<td></td>
<td>R model</td>
<td>0.592 (0.990)</td>
<td></td>
<td></td>
<td>0.004 (0.023)</td>
<td>2.98 (1.39-6.38)</td>
<td></td>
</tr>
</tbody>
</table>

1 Genotype frequencies in the cases and controls, 2 Pearson Chi-squared test. Empirical p value using 5000 permutations, 3 Odds ratios adjusted for asthma status and intake group, 4 Recessive model

**5.5.2 The association between the IL-6 -174G/C and C. pneumoniae antibodies (unpublished results)**

In addition, the association between the IL-6 -174G/C polymorphism and C. pneumoniae antibodies was analysed among the whole study population (n = 867). The minor allele frequency for IL-6 -174G/C was 0.478. Additive, genotypic and dominant models (for the minor allele) as well as multivariate analyses were performed. Under the genotypic, additive and dominant models, the -174C allele was significantly associated with IgG seropositivity on arrival and with persistence of IgG antibodies (Table 16). The association was also significant in the departure samples. Furthermore, in multivariate analysis, the C allele and C/C genotype were independent risk factors for IgG seropositivity and persistent IgG antibodies when BMI, asthma status, intake group and smoking were included in the model (Table 16). The polymorphism was not associated with IgG seroconversion.

In addition, the association between the -174G/C polymorphisms and a slightly elevated CRP level was analysed. The geometric mean and upper quartiles of hsCRP concentration on arrival were 0.83 mg/l and 2.17 mg/l, and at departure, 0.55 mg/l and 1.37 mg/l, respectively. Under the genotypic, additive
and dominant models, the -174C allele was significantly associated with an elevated CRP level at departure (over the upper quartile) (Table 17). In the multivariate model, the C allele and C/C genotype compared with the G/G genotype were independently associated with an elevated CRP level when adjusted for BMI, asthma status, intake group and smoking. The association was parallel, however not significant, on arrival.

To further analyse the association between the -174C/C genotype and persistent infection and inflammation, those who had an elevated CRP level and persistent antibodies during service, and those who had either of them, were compared with those who had neither of them. The odds ratios (95% CI) for the C/C genotype in these groups were significant: 3.45 (2.00–5.98) and 1.41 (1.00–1.99), respectively.

Table 16. Association between the IL-6 -174G/C polymorphism and C. pneumoniae IgG seropositivity and persistent IgG antibodies.

<table>
<thead>
<tr>
<th>IL-6-174 G/C</th>
<th>Frequencies (%)</th>
<th>p value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IgG seropositivity</strong></td>
<td>control/cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>27.9/19.6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>G/C</td>
<td>47.9/46.6</td>
<td>1.31 (0.92-1.86)</td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>24.3/33.8</td>
<td>0.001</td>
<td>1.99 (1.35-2.93)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>51.8/42.9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>48.2/57.1</td>
<td>0.0002</td>
<td>1.40 (1.15-1.69)</td>
</tr>
<tr>
<td>G/G + G/C vs. C/C</td>
<td></td>
<td>0.002</td>
<td>1.52 (1.10-2.11)</td>
</tr>
<tr>
<td><strong>Persistent IgG antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>26.3/18.1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>G/C</td>
<td>48.4/44.2</td>
<td>1.30 (0.85-2.00)</td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>25.2/37.6</td>
<td>0.001</td>
<td>2.25 (1.43-3.54)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>50.5/40.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>49.5/59.7</td>
<td>0.0002</td>
<td>1.52 (1.21-1.90)</td>
</tr>
<tr>
<td>G/G + G/C vs. C/C</td>
<td></td>
<td>0.0005</td>
<td>1.65 (1.11-2.46)</td>
</tr>
</tbody>
</table>

1 Pearson Chi-squared test, 2 Odds ratio (95% confidence interval), adjusted for BMI ≥ 25, asthma status, intake group and smoking, 3 IgG titre ≥ 32 on arrival, 4 IgG antibody persistence in arrival and departure sera
Table 17. Association between the IL-6-174 G/C polymorphism and an elevated hsCRP level (hsCRP level ≥ upper quartile (1.37 mg/l) at departure).

<table>
<thead>
<tr>
<th>IL-6-174 G/C</th>
<th>Frequencies (%)</th>
<th>p value1</th>
<th>OR (95% CI)2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>control/cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>25.7/17.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/C</td>
<td>47.4/46.3</td>
<td>0.016</td>
<td>1.41 (0.90-2.22)</td>
</tr>
<tr>
<td>C/C</td>
<td>26.8/36.2</td>
<td></td>
<td>1.95 (1.21-3.15)</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>49.5/40.7</td>
<td>0.003</td>
<td>1.40 (1.11-1.77)</td>
</tr>
<tr>
<td>C</td>
<td>50.5/59.3</td>
<td></td>
<td>1.61 (1.05-2.46)</td>
</tr>
<tr>
<td>G/G + G/C vs. C/C</td>
<td>0.014</td>
<td>1.61 (1.05-2.46)</td>
<td></td>
</tr>
</tbody>
</table>

1 Pearson Chi-squared test, 2 Odds ratio (95% confidence interval), adjusted for BMI ≥ 25, asthma status, intake group and smoking

5.6 Association between both C. pneumoniae antibodies and hsCRP levels and an elevated BMI (IV)

The study population was divided into two groups according to BMI: normal-weight (BMI < 25 kg/m²; n = 552) and overweight conscripts (BMI ≥ 25 kg/m²; n = 339). There were 92 obese conscripts with a BMI ≥ 30, who were included in the overweight group. Overweight conscripts were slightly older than normal-weight conscripts (p = 0.036). There was no statistically significant association in asthma or smoking between the BMI groups (p = 0.501 and 0.179, respectively). Education was associated with BMI; the conscripts with a comprehensive school or vocational education were more often overweight than were those with an upper secondary school education (p < 0.001). IgG seropositivity (≥ 32) on arrival was significantly more prevalent among overweight than among normal-weight conscripts (54% vs. 41%, p < 0.001). Persistent IgG antibodies were significantly associated with overweight (p < 0.001), whereas IgG seroconversion was not (p = 0.440) (study IV, Table 1).

The geometric mean hsCRP concentration on arrival was 0.84 mg/l. The difference in the mean CRP concentration between normal-weight and overweight conscripts was significant (0.69 mg/l vs. 1.16 mg/l; p < 0.001). The upper quartile of CRP concentration in the arrival and departure samples was 1.70 mg/l. A persistently slightly elevated CRP level (defined as a CRP level ≥ the upper quartile on arrival and at departure) was associated with the overweight group (p = 0.007) (study IV, Table 1). In addition, a positive correlation between BMI and
hsCRP on arrival ($r = 0.234$, $p < 0.001$) and *C. pneumoniae* IgG titre ($r = 0.130$, $p < 0.001$) was observed.

In the logistic regression analysis, an elevated CRP level on arrival, a persistently high CRP level, *C. pneumoniae* IgG seropositivity, and persistent IgG antibodies proved to be significant risk factors of overweight when adjusted for intake group and education (Table 18). In addition, a significant risk was observed when those who had a persistently elevated CRP level and persistent antibodies during service, and those who had either of them, were compared with those who had neither of them (Table 18). Adjusting the analyses for asthma status and smoking did not alter the results. In addition, statistically significant trends were found between the normal-weight, overweight, and obese groups; $p < 0.001$ for an elevated CRP level, $p = 0.001$ for a persistent CRP level, $p = 0.009$ for IgG seropositivity, $p < 0.001$ for persistent IgG and $p < 0.001$ for combined persistent CRP and persistent antibodies.

BMI values together with co-variances were analysed in the different groups. Asthmatic status, smoking, intake group and education were included in the model. Asthma status and smoking were not statistically significant and were excluded from the final model. Table 18 shows the geometric mean BMI values for the categorised hsCRP levels and *C. pneumoniae* IgG antibodies.
### Table 18. Odds ratios for overweight (BMI ≥ 25) and mean values of BMI in different groups of hsCRP level and *C. pneumoniae* IgG antibodies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n (%)</th>
<th>OR (95% CI)</th>
<th>BMI kg/m², mean (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP negative</td>
<td>168 (32)</td>
<td>1</td>
<td>23.5 (23.2-23.8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CRP positive</td>
<td>110 (49)</td>
<td>2.0 (1.4-2.7)</td>
<td>25.0 (24.5-25.5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>persistent CRP negative</td>
<td>206 (34)</td>
<td>1</td>
<td>23.8 (23.5-24.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>persistent CRP positive</td>
<td>28 (54)</td>
<td>2.2 (1.3-3.9)</td>
<td>25.6 (24.5-26.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cpn IgG negative</td>
<td>147 (33)</td>
<td>1</td>
<td>23.7 (23.3-24.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cpn IgG positive</td>
<td>167 (44)</td>
<td>1.5 (1.1-2.0)</td>
<td>24.5 (24.1-24.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cpn persistent IgG negative</td>
<td>165 (32)</td>
<td>1</td>
<td>23.7 (23.4-24.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cpn persistent IgG positive</td>
<td>106 (49)</td>
<td>2.1 (1.5-2.8)</td>
<td>24.8 (24.3-25.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cpn IgG persistent and CRP persistent negative</td>
<td>123 (29)</td>
<td>1</td>
<td>23.5 (23.1-23.8)</td>
<td></td>
</tr>
<tr>
<td>Cpn IgG persistent or CRP persistent</td>
<td>101 (47)</td>
<td>2.2 (1.6-3.2)</td>
<td>24.6 (24.2-25.1)</td>
<td></td>
</tr>
<tr>
<td>Cpn IgG persistent and CRP persistent positive</td>
<td>10 (56)</td>
<td>3.0 (1.2-7.9)</td>
<td>25.8 (24.1-27.6)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

1 Adjusted for intake group and education, 2 p value for the difference between geometric mean values of BMI, 3 hsCRP level ≥ upper quartile (1.7 mg/l) on arrival, 4 hsCRP level ≥ upper quartile on arrival and at departure (1.7 mg/l), 5 IgG titre ≥ 32 on arrival, 6 IgG positive in arrival and departure sera.

*Cpn, Chlamydia pneumoniae; CRP, C-reactive protein*
6 Discussion

6.1 Methodological considerations and limitations of the study

6.1.1 Study population

All the studies in this work were performed in the same study population, which included 893 Finnish military recruits from the military service in Kainuu Brigade, in northern Finland. The studies were prospective nested case-control studies. The questionnaire data were collected at the beginning of their service, respiratory tract infections were followed during their service and serum samples were collected at the beginning and end of their service and during each infectious episode. This material gave a good opportunity to do a frequent follow-up analysis during the six to twelve months of service and enabled the study of respiratory tract infectious episodes and *C. pneumoniae* exposure as well as persistence of *C. pneumoniae* antibodies during that time.

Military service in Finland is mandatory and all men aged 18–19 years are called up for service. Ninety-eight percent of them attend a call-up examination to establish their fitness for military service (Latvala et al. 2005). Altogether 80–85% of all men, including over 80% of asthmatic young men, complete their service (Kajosaari 2004). Therefore, this study population represents well the healthy population of young Finnish men. The study population consisted exclusively of Finns who came mainly from the same part of Finland, thus representing an ethnically homogenous population.

The study population included 229 military recruits with asthma that was previously defined by a physician according to their own opinion. In addition, the diagnosis was confirmed from previous medical examinations. The diagnostic criteria used were not available. All the asthma cases were rather mild, because those with serious asthma were exempted at the call-up examinations or were discharged at the beginning of their service. Conscripts with asthma were more prone to respiratory tract infections than those without during 180-day military service. This was also shown by Juvonen et al. (2008b), who studied the risk factors for acute respiratory tract illness in this same study population of military recruits in 180-day service. It is well known that people with asthma are more susceptible to respiratory tract infections than are people without asthma and that respiratory tract infections are important inducers of asthma exacerbations.
In addition, respiratory tract infections were more common in the January 2005 intake group than in the July 2004 intake group. There were also differences in the presence of *C. pneumoniae* antibodies between intake groups. These factors were taken into account by adjusting the multivariate analyses of infectious episodes and *C. pneumoniae* for asthma status and intake group.

Increased IL-6 and sIL-6R levels are also known to be associated with asthma (Doganci *et al.* 2005, Yokoyama *et al.* 1997). However, we did not find any association between *IL-6* or *IL-6R* gene polymorphisms and asthmatic status in our study group. Neither did we find any association between MBL and asthma or that the effect of MBL deficiency would be dependent on asthmatic status. On the other hand, the most serious asthma cases were not included, and the number of asthmatic subjects was also quite low. Since there was no difference in the *MBL2*, *IL-6* or *IL-6R* genotype distributions between men with asthma and those without, the infection associations could be considered in all men together.

The study population was not determined for the purposes of genetic analyses and therefore, the presented genetic studies are exploratory studies. The number of study participants was rather small and especially the power for finding genetic associations for polymorphisms that have a small effect was low. In studies I and III, genetic susceptibility to respiratory tract infections was studied only among men in 180-day service. These men served only in Kainuu Brigade and completed the follow-up. Post-study power calculations indicated that especially in the *MBL2* studies (I and II), the power to detect genetic associations was too low (15% to 30%). Study I consisted of only the January 2005 intake group, which included more infectious episodes during service than the July 2004 intake group, probably due to the winter period. The power for detecting associations decreased when the two intake groups were combined. For the *IL-6* and *IL-6R* analysis (III) the power was nearly sufficient if 80% was considered the limit. In study IV, the *C. pneumoniae* seroprevalence in different groups was high and the power was over 80%. As the study population included only men, there may be a gender bias in these studies and the results cannot be generalised in the female population. Therefore, it would be interesting to replicate these studies in a larger cohort.

### 6.1.2 Data on respiratory tract infections

Respiratory tract infections are a major cause of sick days during military service. Military trainees are at a high risk of respiratory epidemics because of their
crowded living conditions, stressful working environment, frequent travel, and exposure to novel strains of respiratory pathogens (Gray et al. 1999). Training during the winter months, when most viral epidemics occur, further predisposes military recruits to respiratory tract infections. The living conditions in military service could be compared to kindergartens, where infections spread easily. The high infection load present during military service may help us easily pick out the persons most susceptible to infection and make analysis of the potential association easier.

In this study, respiratory tract infections were diagnosed at the primary care clinic in Kainuu Brigade. Infections were included in the data only if diagnosed by a physician. Mild episodes not referred to a physician were not included and therefore minimally symptomatic or asymptomatic infections are probably missing from the data. Furthermore, infections during the conscripts’ time at home, e.g. during weekends, are missing. Approximately half of the conscripts had no diagnosed episodes during service. They probably experienced only mild episodes. The majority of the respiratory infections were diagnosed as upper respiratory tract infections (> 90%). The etiological results of this study population will be published in more detail in the near future.

6.1.3 Selected gene polymorphisms and genotyping methods

The aim of this study was to investigate genetic variations in the genes of molecules important in innate immunity. These candidate genes were selected on the basis of current knowledge from the literature and the personal interests of our study group.

Six known SNPs were selected from the MBL2 gene (studies I and II). These polymorphisms have been widely studied in the last few years and associated with different kinds of infections. However, very little information on the association between MBL and respiratory tract infections has been published. We used a previously developed real-time PCR method on a LightCycler instrument for MBL2 genotyping. The method enabled rapid detection of all six polymorphisms using three capillaries and two protocols. With the addition of melting curve analysis in the LightCycler system, the method was an efficient tool for mutation detection, and distinguishing two alleles was easy. In addition, sequenced samples confirmed the PCR results.

SNPs of the IL-6 and IL-6R genes were carefully selected after evaluation of the literature and NCBI and by including some tag SNPs that capture other SNPs
(study III). The selected SNPs have been previously associated mainly with metabolic disorders and cardiovascular diseases. Only a few studies have been published on the association between \textit{IL-6} -174G/C and respiratory tract infections. Genotyping was performed by MALDI-TOF mass spectrometry in Karolinska Institute from where the quality-controlled results were reported.

In addition, all the allele frequencies were in Hardy-Weinberg equilibrium, indicating that systemic genotyping errors were unlikely.

\textbf{6.1.4 Measurement of serum MBL concentrations}

Serum MBL levels were measured with a commercial ELISA test according to the manufacturer’s instructions (Human MBL Lectin assay, studies I and II). The test measures functional MBL that is captured by solid bound mannann in microtiter wells. It has been shown that \textit{MBL2} polymorphisms may express aberrant MBL that has no detectable functionality. Therefore, it is possible that aberrant MBL was not detected by our test. Frederiksen \textit{et al.} (2006) compared the quantification of MBL by four commercially available assays and four of their own in-house assays. They showed a correlation between the assays in \textit{MBL2} haplotypes that are usually associated with high MBL levels, but there was significant variation in the assays in \textit{MBL2} haplotypes that are associated with low MBL levels. Mainly the assays that use a sandwich-type protocol (antibody-coated surface) measured higher levels than those that used a solid-phase mannann-coated surface. The findings indicate that specific antibodies are able to detect the aberrant forms of MBL found in individuals carrying \textit{MBL2} variant alleles, whereas mannann-coated assays are not able to. However, comparable results were obtained with all the assays. Regardless of the variation in assays that are used in different populations, comparable proportions of MBL-deficient individuals are obtained.

In the current work, some inaccuracy may have been occurred since the ELISA test was multiphased and quite complicated and only one measurement per sample was done. To minimise variation between tests, the MBL concentrations were measured by one person and the tests were monitored by running the same controls in each test. The aim was to obtain MBL levels comparable with each other.
6.1.5 Determining persistent C. pneumoniae antibodies

Serum C. pneumoniae antibodies were measured with the MIF method, which is considered the golden standard for serological diagnosis of chlamydial infections (Dowell et al. 2001). However, there is large interlaboratory variation in antibody titres with the subjective MIF method (Peeling et al. 2000) and a lack of reliable serological markers for chronic infection (Dowell et al. 2001). In the current work (studies II, IV and some unpublished results) we only analysed the presence or persistent presence or fourfold titre changes in C. pneumoniae antibodies. Experienced laboratory technicians performed the assays and the results were read by one experienced reader. In addition, our laboratory has long-term experience in the use of MIF assays and is one of the international reference laboratories for MIF tests. The presence of IgG and/or IgA serum antibodies is a marker of past exposure to C. pneumoniae. It is known that even IgG antibodies do not normally persist in young persons (Paldanius et al. 2007), and thus persistence of antibodies might also indicate persistence of infection. It has been suggested that the presence of short-living IgA antibodies might be a better indication of chronicity than are IgG antibodies. In this study population, persistence of IgA antibodies were associated with BMI (p = 0.010), but due to the low number of participants with IgA seropositivity in this young study population, persistent IgA antibodies were not reported.

6.2 Main findings

6.2.1 Serum MBL levels and MBL2 genotypes in the study population

The serum MBL levels and MBL2 genotypes in the January 2005 intake group were presented in study I, but the data from both intake groups are shown here. The results were similar in both intake groups. The MBL2 genotype frequencies of this study population were similar to those previously observed in Caucasian and Finnish populations (Aittoniemi et al. 2005, Garred 2008). The B allele was the most common and the C and D alleles were more rare structural variant alleles.

The median serum MBL concentration was 1156.5 ng/ml, which is comparable with other populations: the median levels were 1550 ng/ml in Australian blood donors (Minchinton et al. 2002) and 992 ng/ml in Danish blood donors (Garred et al. 1992). However, in an earlier study, healthy Finnish adults had a median MBL level of even 4002 ng/ml (Aittoniemi et al. 1996). An
explanation for the great difference between these two Finnish populations could be in the variations in the methodology.

Earlier studies have shown that the promoter polymorphism and three exon 1 polymorphisms (variant alleles D, B and C) of the MBL2 gene have an effect on MBL protein levels. L and X alleles have been described to correlate with low MBL levels and the effect of the X allele has been the most significant (Madsen et al. 1995, Minchinton et al. 2002). In a study of a Korean population, the effect of an X/Y polymorphism was not significant and L and P alleles were significantly correlated with low MBL levels (Lee et al. 2005). Similar observations were made in this study population. The results suggested that the Y/Y genotype would be associated with higher MBL levels than the X/Y and X/X genotypes, but the difference was not significant. L and P alleles were significantly associated with lower MBL levels.

The structural variant alleles seemed to have the most significant effect on MBL levels. The wild-type genotype A/A correlated with very high serum MBL levels, the heterozygous variant genotype A/O with intermediate levels and the homozygous variant genotype O/O with almost undetectable MBL levels. This kind of distribution has also been reported earlier (Garred 2008, Garred et al. 2006). The frequency of MBL deficiency defined as the O/O genotype (practically below the detection limit) was 4.8%. The proportion was very similar to those found in similar ethnic groups (Aittoniemi et al. 1996, Dahl et al. 2004).

6.2.2 MBL and respiratory tract infections (I)

Study I investigated an association between MBL levels, MBL2 gene polymorphisms and respiratory tract infections and found that a below-median MBL level was a significant risk factor for infections. In the earlier studies by Aittoniemi et al. (1998) and Thorarinsdottir et al. (2005), MBL serum levels did not differ between children with repeated respiratory tract infections and healthy individuals, but Chen et al. (2009) found that MBL levels were significantly lower in children with recurrent respiratory tract infections compared with healthy controls. Recently, it was shown in an adult Caucasian population that MBL deficiency (defined as ≤ 50 ng/ml) was significantly more frequent in patients with recurrent and/or severe infections and furthermore, the association was significant also when only respiratory tract infections were included (Hoeflich et al. 2009). We did not find any association for MBL deficiency (defined as O/O
genotype), possibly due to the low proportion of this allele in our study population.

There were more men with the A/O or O/O genotype than with the A/A genotype who had at least two infectious episodes during their service. This difference was not significant, but after adjustment for asthma status, a borderline significant risk of 1.7-fold for men with the A/O or O/O genotype was detected. Furthermore, a clear association between the groups with high and low MBL concentrations (below or above the median) and the \textit{MBL2} structural genotype groups A/A and A/O + O/O was demonstrated, suggesting that the low-producing \textit{MBL2} exon 1 genotypes should also affect susceptibility to respiratory tract infections. It seems that there may be an association between exon 1 variant alleles and respiratory tract infections, but evidently due to the low power in this study, a significant association was not found. Two studies have reported an association between \textit{MBL2} polymorphisms and respiratory tract infections among adults, with contradictory results (Dahl \textit{et al.} 2004, Gomi \textit{et al.} 2004). Gomi \textit{et al.} found an association between the structural variant allele B and recurrent respiratory tract infections among adult patients, but Dahl \textit{et al.} found no association among adults in a large population-based study. However, there are several studies on \textit{MBL2} variants and respiratory tract infection susceptibility among children, and most of them identify the A/O and O/O genotypes as risk factors for infections (Cedzynski \textit{et al.} 2004, Chen \textit{et al.} 2009, Koch \textit{et al.} 2001, Summerfield \textit{et al.} 1997, Wiertsema \textit{et al.} 2006a).

In addition, the promoter genotype Y/Y was a significant risk factor for infections. Furthermore, the Y/Y genotype and the exon 1 variant alleles had a significant combined effect on respiratory tract infections. It is possible that there is some interaction between the Y allele and the exon 1 variant alleles, but this requires further study.

Previous studies on the association between \textit{MBL2} variants and respiratory tract infections have included patients or immunodeficient subjects with severe respiratory tract infections requiring hospitalisation or antibiotic treatment. This healthy, young Finnish population experienced mainly moderately severe respiratory tract infections requiring consultation by a physician. Thus, these findings suggest that low MBL levels and \textit{MBL2} polymorphisms predispose not only to serious infections, especially in immunodeficient patients, but also to common respiratory infections in otherwise healthy people.
6.2.3 MBL and C. pneumoniae infection (II)

Study II presented a direct association between MBL and C. pneumoniae infection in vivo, to our knowledge, for the first time. The exon 1 variant allele genotypes (A/O and O/O) and Y/Y genotype were associated with the presence of elevated C. pneumoniae IgG or IgA antibodies, indicating past exposure to C. pneumoniae. In addition, an MBL level below the median concentration and the P/P genotype were associated with C. pneumoniae seroconversions that indicate clinical or subclinical infections during military service. Interestingly, low serum MBL levels were significantly associated with susceptibility to C. pneumoniae infection during military service, but not with the presence of elevated antibodies, suggesting that low MBL levels do predispose to infection susceptibility, but possibly not to development of chronic infection. The results of these two analyses are in agreement with previous studies and with our findings on MBL and respiratory tract infections, where the exon 1 variant allele genotypes and a below-median MBL level have been associated with respiratory tract infections.

In vitro studies by Swanson et al. (1998) have shown that MBL inhibits cell culture infection by Chlamydia. In addition, several other studies have reported the structure of the carbohydrates of the outer membrane of chlamydia, their role in the infectivity of host cells (Kuo et al. 1996, Swanson & Kuo 1991, 1994) and also the ligands that inhibit infection of host cells by chlamydia (Kuo et al. 2007, Puolakkainen et al. 2005). It is possible that MBL binds to the mannose structures located on the outer membrane of chlamydia and blocks attachment and entry of the organism into the host cell. This possibility was investigated and suggested by Swanson et al. (1998).

A direct association between MBL and C. pneumoniae antibodies has not been indicated before. Rugonfalvi-Kiss et al. (2002) studied the role of MBL in the association between C. pneumoniae and coronary artery disease. The association was found only among those carrying MBL2 variant alleles and not in those homozygous for the wild-type allele. They concluded that C. pneumoniae infection leads to the development of severe coronary artery disease mainly in those with the MBL2 exon 1 variant allele. Structural MBL2 variant alleles have been associated with atherosclerosis and cardiovascular diseases in a few studies, and it has been suggested that MBL deficiency could predispose the host to C. pneumoniae infection, which, in turn, may play a role in the development of atherosclerosis (Jowett 2010, Madsen et al. 1998b). Furthermore, Nagy et al. (2003) have reported that MBL2 exon 1 variant alleles have an important role in
susceptibility to asthma in children infected with *C. pneumoniae*. They did not observe a direct association between *MBL2* genotypes and *C. pneumoniae* antibodies. Both of these studies found an association between *C. pneumoniae*, the *MBL2* variant allele and a chronic inflammatory disease, suggesting that *MBL2* genetics may have a role behind the association between *C. pneumoniae* and inflammatory disease and that MBL plays an important role in the immune response against *C. pneumoniae* infection.

### 6.2.4 IL-6 and IL-6R gene polymorphisms and respiratory tract infections (III)

Study III investigated the association between polymorphisms in the *IL-6* and *IL-6R* genes and susceptibility to respiratory tract infections during military service. The promoter polymorphism IL6R1 (-183G/A) and two intron 1 polymorphisms, IL6R2 and IL6R3, were found to be significant risk factors for infections.

The *IL-6* -174C/G polymorphism has been associated before with the risk for upper respiratory tract infections and with susceptibility to otitis media in children aged 6–35 months (Patel *et al.* 2006, Revai *et al.* 2009). Here the IL6A G/G genotype was associated with infections only in combination with the IL6R1 A/A genotype. This may suggest that the *IL-6* gene polymorphism would associate with infections in combination with the *IL-6R* polymorphism rather than independently.

Exon 9 polymorphism IL6R5 (48892A/C) has been connected with higher plasma CRP levels and IL-6 levels (Qi *et al.* 2007, 2009), elevated BMI, type 2 diabetes risk and metabolic syndrome (Esteve *et al.* 2006, Hamid *et al.* 2004, Wolford *et al.* 2003), and risk of periodontitis (Galicia *et al.* 2006). Here we did not find any association between IL6R5 and respiratory tract infections. Promoter polymorphism IL6R1 (-183G/A) has been connected before with elevated BMI and obesity (Bustamante *et al.* 2007). The strongest associations with a risk of respiratory tract infections were found for the IL6R1 and IL6R2 polymorphisms, which were in the same LD block. A significant risk was obtained for one or more infections as well as with frequent infections (3 or more), thus strengthening the association. In addition, IL6R3 from intron 1 was associated with frequent infections. Previous and current studies may suggest that variation in the 3′ end of the *IL-6R* gene would be associated with obesity and metabolic disorders, whereas the 5′ area of the gene may be associated with respiratory tract infections.
The promoter polymorphism IL6R1, located in a transcription factor binding site of the gene, could have an effect on gene transcription (Kim et al. 2003). The mechanism with which this mutation affects activation or repression of transcription is not clear. Galicia et al. (2004) reported in their study of 70 healthy adult Japanese that -183A allele carriers had higher sIL-6R levels compared with G allele carriers. In our study, the A allele was associated with infections. Possibly the A allele induces transcription of a soluble form of the receptor and then sIL-6R acts as an IL-6R agonist, blocking IL-6 from binding to cell-membrane-bound IL-6R. This may further decrease the activation of leucocytes and thus have an influence on the immune response against infection.

sIL-6R is produced by alternative splicing of mRNA or by proteolytic cleavage (Mullberg et al. 1994). It has been shown that the product of alternatively spliced mRNA is a predominant form of sIL-6R, especially in youngsters. It is also known that a release of those two forms is differentially regulated in specific disease states (Jones et al. 2001b). Possibly, if the promoter and intronic SNPs presented here would have a regulatory role in mRNA splicing, the regulation of the alternatively spliced form of sIL-6R could play a predominant role in susceptibility to infections in young men.

Associations between IL-6R gene polymorphisms and respiratory tract infections have not been reported before and therefore, these findings are preliminary. However, the associations were strong and encourage replication to confirm our observations.

6.2.5 IL6 -174G/C polymorphism and C. pneumoniae (unpublished results)

The IL-6 -174C allele and the C/C genotype were associated with C. pneumoniae IgG seropositivity and persistent IgG antibodies. However, IL-6 genotypes were not associated with IgG seroconversion, a marker of acute infection during military service. These findings point to the possibility that the C allele is associated with prolonged or chronic C. pneumoniae infection. In addition, the C allele and C/C genotype were also significantly associated with a slightly elevated CRP level, a marker of systemic inflammation, and furthermore, when persistent C. pneumoniae antibodies were present together with elevated CRP, the association was clearly stronger than when either of them was positive alone, suggesting that the C allele is linked to chronic infection with systemic low-grade inflammation.
Previous results on the association between the IL-6 genotypes and plasma levels have been inconsistent (Fishman et al. 1998, Jones et al. 2001a). However, high levels of IL-6 (Bennet et al. 2003, Jones et al. 2001a, Ridker et al. 2000b) and the -174C allele (Georges et al. 2001, Hulkkonen et al. 2009, Humphries et al. 2001, Licastro et al. 2004) have been connected with cardiovascular diseases and risk factors of atherosclerosis. The association between C. pneumoniae antibodies and IL-6 has not been reported before. C. pneumoniae infection has been shown to induce production of cytokines, such as IL-6, in activated monocytes and macrophages (Kaukoranta-Tolvanen et al. 1996). It could be hypothesised that in persistent C. pneumoniae infection, production of IL-6 is persistently induced, leading to continuous production of CRP, especially in persons with the -174C allele genotype. Possibly, elevated plasma IL-6 levels and CRP would then play a role in the pathogenesis of atherosclerosis.

In a recent Finnish study of a cohort of young men aged 24–39 years, the -174G/C polymorphism was associated with the risk factors and markers of atherosclerosis (Hulkkonen et al. 2009). They found an association between the genotypes and serum HDL level and apoA1 level but not with CRP level or BMI.

In addition, persistent C. pneumoniae seropositivity has been associated with increased aortic intima-media thickness already in 11-year-old healthy children, suggesting that C. pneumoniae may play a role in the pathogenesis of early atherosclerosis (Volanen et al. 2006). Therefore, our study could suggest that IL-6 genetics may mediate a risk for cardiovascular diseases already at a young age, whereas previously the association has been shown mainly in an older population.

### 6.2.6 C. pneumoniae and elevated BMI (IV)

In study IV, C. pneumoniae IgG antibodies and slightly elevated CRP levels were significantly associated with overweight (BMI ≥ 25). Especially, an association was detected for persistent IgG antibodies and persistently high CRP levels and, furthermore, for the combined presence of persistent antibodies and CRP, suggesting prolonged or chronic infection. Interestingly, antibody seroconversion during military service, indicating a possible acute C. pneumoniae infection, was not associated with BMI at all.

C. pneumoniae has been associated with obesity also earlier (Dart et al. 2002, Ekesbo et al. 2000, Karppinen et al. 2003, Lajunen et al. 2008a, Thjodleifsson et al. 2008). However, there are a few studies that did not find any association (Kaftan & Kaftan 2000, Koziolek et al. 2008). In addition, the interaction
between *C. pneumoniae* and changes in lipid metabolism has been shown (Laurila et al. 1997b, Leinonen 2000, Leinonen & Saikku 1999). In earlier studies mainly single-point antibody measurements have been used to indicate earlier exposure to infection. However, here the serum samples taken six to twelve months apart were available and enabled studying persistently elevated CRP concentrations as well as persistent *C. pneumoniae* antibodies during service.

Also several viruses in animals and two viruses, SMAM-1 avian adenovirus and human adenovirus Ad-36, in humans have been connected with obesity (Atkinson et al. 2005, Dhurandhar 2001, Dhurandhar et al. 1997). Ad-36 is able to infect adipocytes and it has been suggested that Ad-36 has a direct effect on adipogenesis and lipid accumulation by adipocytes (Dhurandhar et al. 2000, Vangipuram et al. 2004). Recently, it was shown that also *C. pneumoniae* is able to infect murine pre-adipocytes and adipocytes (Shi et al. 2008) as well as human adipocytes (Bouwman et al. 2008). *C. pneumoniae* promotes the secretion of inflammatory molecules, such as TNF-α, and thus, more likely suppresses insulin signalling and adipogenesis by inflammatory mechanisms (Shi et al. 2008). *C. pneumoniae* is able to infect macrophages and induce lipid accumulation and foam cell formation in them (Kalayoglu & Byrne 1998a). It may possibly get into adipose tissue by infected pulmonary macrophages that then induce lipid accumulation and induce the production of proinflammatory molecules in adipose tissue. Macrophages produce the same proinflammatory molecules, IL-6 and TNF-α, as adipose tissue, and *C. pneumoniae* has been shown to induce the production of these molecules from infected macrophages (Kaukoranta-Tolvanen et al. 1996).

The causality between infections and obesity has not been resolved. In this study, the rate of *C. pneumoniae* seroconversion was similar in overweight and normal-weight subjects. This suggests that obese subjects would not be more susceptible to acute *C. pneumoniae* infections, but that possible persistent *C. pneumoniae* infection predisposes to overweight or that overweight may induce the chronicity of *C. pneumoniae* infection. Increased IL-6 production in obesity induces CRP production and further low-grade inflammation. Inflammation then may impair individuals’ immune response, predisposing them to infections and persistence of infection. The findings that weight loss has a decreasing effect on IL-6 and CRP levels, reflecting advantageous effects on inflammation (Eklund et al. 2006, Esposito et al. 2003, Viljanen et al. 2009), support this causality. However, it has been shown that there are obese individuals that are metabolically healthy. Barbarroja et al. (2010) suggested that the obesity-associated
development of metabolic complications depends on the degree of inflammation of the adipose tissue. The hypothesis was supported by the findings that insulin-resistant obese individuals had increased macrophage infiltration, increased levels of IL-1β and IL-6 and increased activation of proinflammatory signalling compared with equally obese but insulin-sensitive individuals. In addition, Makkonen et al. (2007) reported that increased macrophage infiltration and expression of inflammatory markers were correlated with liver fat content, which is associated with insulin resistance, independently of obesity. These studies raise a question: what causes the inflammation in adipose tissue? Could an infection be the trigger? The causality between infections and obesity could also be bidirectional. Overweight may predispose to infections, and if the infectious agents then reach adipose tissue they may further induce weight gain.

The previous studies have been mainly conducted among older and CHD patient populations (Dart et al. 2002, Ekesbo et al. 2000, Karppinen et al. 2003, Lajunen et al. 2008a), whereas these results suggest that infections could be a risk factor of overweight already at a young age, or vice versa. Thus, our results strongly point to the possibility that both persistent infection and systemic inflammation are associated with obesity in these young men.

6.3 Future prospects

We showed here that MBL2 and IL-6R gene polymorphisms were associated with respiratory tract infections, in general. In the future, it would be interesting to study if these polymorphisms are associated with specific etiological agents, including e.g. adenovirus, rhinovirus and influenza A and B viruses, which are known to cause respiratory tract infections in young men. During our study we did not have any data on viral etiology available.

We also found an association between MBL concentration, MBL2 gene polymorphisms and C. pneumoniae antibodies. Similar findings have not been reported before and therefore, they should be repeated. It has been shown earlier that MBL protein is able to inhibit C. pneumoniae infection of HeLa cells (Swanson et al. 1998) and, thus, further studies are required to investigate the pathogenetic mechanisms by which MBL protects from chlamydial infection.

We did not measure IL-6 concentration in this study population. There are controversial reports on the effect of IL-6 polymorphisms on IL-6 levels. In the future, serum IL-6 concentrations should be measured in this study population.
and the interaction of IL-6 levels with IL-6 genotypes, respiratory tract infections and *C. pneumoniae* antibodies should be studied.

It is currently well known that infections are associated with obesity. However, the causality is not resolved yet. The present study showed that persistence of *C. pneumoniae* antibodies and elevated CRP levels, suggesting chronic infection, were associated with overweight, but did not prove any causality. Studies on the pathogenetic mechanisms behind this association are needed. *In vitro* cell cultures would provide the possibility to investigate e.g. *C. pneumoniae* infection in pre-adipocytes and adipocytes and infection-induced cytokines and other factors associated with obesity.
7 Summary and conclusions

The following results were obtained for the specific study questions:

1. A significant variation in MBL levels between different MBL2 genotypes and an MBL deficiency in homozygous exon 1 variant allele genotype (O/O) were demonstrated. MBL2 genotype frequencies and MBL levels were comparable with other populations.

2. A low MBL level was a significant risk factor for susceptibility to respiratory tract infections. Among the six MBL2 SNPs, there was a significant association between the promoter genotype Y/Y (-221) and infections and a borderline significant association between exon 1 variant allele genotypes and infections.

3. A low MBL level and certain MBL2 polymorphisms were significantly associated with elevated C. pneumoniae antibodies and seroconversions, suggesting that MBL is associated with susceptibility to C. pneumoniae infection.

4. The IL-6R promoter polymorphism -183G/A and two intron 1 polymorphisms were significantly associated with respiratory tract infections. The IL-6 gene polymorphism -174G/C was associated with infections only in combination with an IL-6R polymorphism.

5. The IL-6 -174G/C polymorphism was significantly associated with persistence of elevated C. pneumoniae IgG antibodies. The risk was further increased when present together with slightly elevated CRP levels, pointing to chronic C. pneumoniae infection.

6. Persistence of elevated C. pneumoniae IgG antibodies as a suggestive marker of chronic infection, and slightly elevated CRP levels as a marker of systemic inflammation, were significantly associated with an elevated BMI.

The study population of this work included healthy, young Finnish men. These findings support the role played by MBL in susceptibility to infections in earlier reports and provide new information about the association between MBL and common respiratory tract infections.

New associations between variations in the IL-6R gene, a receptor of one important proinflammatory cytokine, and respiratory tract infections were found. The results suggest that the 5’ area of the IL-6R gene may be a possible candidate region for respiratory tract infection susceptibility. Our finding that the IL-6 -174C allele is associated with persistence of C. pneumoniae antibodies and
elevated CRP levels suggest that persistent *C. pneumoniae* infection promotes IL-6 production, especially in individuals with the *IL-6*-174C allele genotype. Interestingly, elevated IL-6 and CRP levels and *C. pneumoniae* infection have all been previously connected with cardiovascular diseases.

In addition, our findings on the association between *C. pneumoniae* and elevated BMI in this homogenous, young, healthy population confirm previous, less-controlled studies in older populations with more confounding factors. However, this study did not resolve the causality between infection and obesity.

Polymorphisms in immune response genes are known to be associated with susceptibility to infections and may alter the response to infection. However, a statistically significant association in a single study does not guarantee a genetic association, but neither does a lack of statistical significance exclude a possible association (Ioannidis et al. 2001).
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