Major histocompatibility complex and coronary artery disease: Special emphasis on *Chlamydia pneumoniae*, and periodontitis

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Academic Dissertation

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“Satisfaction lies in the effort, not in the attainment. Full effort is full victory.”

Mahatma Gandhi (1869 - 1948)

CONTENTS

1. LIST OF ORIGINAL PUBLICATIONS

This Thesis is based on the following original articles referred to in the text by Roman numerals I-IV.

I. Palikhe A, Sinisalo J, Seppänen M, Valtonen V, Nieminen MS, Lokki M-L. Human MHC region harbors both susceptibility and protective haplotypes for coronary artery disease. *Tissue Antigens* 2007;69(1):47-55.

II. Palikhe A, Lokki M-L, Saikku P, Leinonen M, Paldanius M, Seppänen M, Valtonen V, Nieminen MS, Sinisalo J. Association of *Chlamydia pneumoniae* infection with HLA-B*35 in patients with coronary artery disease. *Clin Vaccine Immunol* 2008;15(1):55-59.

III. Palikhe A, Lokki M-L, Pussinen PJ, Paju S, Ahlberg J, Asikainen S, Seppänen M, Valtonen V, Nieminen MS, Sinisalo J. Lymphotoxin alpha LTA+496C allele is a risk factor for periodontitis in patients with coronary artery disease. *Tissue Antigens.* 2008 Apr 2; [Epub ahead of print].

IV. Palikhe A, Sinisalo J, Seppänen M, Haario H, Meri S, Valtonen V, Nieminen MS, Lokki M-L. Serum complement C3/C4-ratio, a novel marker for recurrent cardiovascular events. *Am J Cardiol* 2007;99(7):890-895.

 This Thesis may include results which are not mentioned in the original publications.

2. ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
ACS	Acute coronary syndrome
ANOVA	Analysis of variance
APC	Antigen presenting cell
Bf	Complement factor B
C. pneumoniae	*Chlamydia pneumoniae*
C2	Complement component C2
C3	Complement component C3
C3/C4 ratio	Ratio of complement component C3 and C4 concentrations
C4	Complement component C4
C4*Q0	Presence of either C4A*Q0 or C4B*Q0
C4A	Complement component C4A (Rodgers blood group)
C4A*Q0	C4A quantitative null allele
C4B	Complement component C4B (Chido blood group)
C4B*Q0	C4B quantitative null allele
CAD	Coronary artery disease
CD	Cluster of differentiation
CD40L	CD40 ligand
CI	Confidence interval
CIITA	Class II MHC transactivator
HLA	Human leukocyte antigen
HTx	Heart transplantation
IC	Immune complex
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
LDL	Low density lipoprotein cholesterol
LGALS2	Lectin galactoside-binding soluble 2
LTA	Lymphotoxin alpha (TNF superfamily member 1)
MHC	Major histocompatibility complex
MHC2TA	MHC transactivator class II
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NS	Non significant

OR	Odds ratio
oxLDL	Oxidized LDL
P. gingivalis	*Porphyromonas gingivalis*
RCCX	Genetic module of RP-C4-CYP21-TNX
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th1	T helper cell type 1
Th2	T helper cell type 2
Thr	Threonine
TLR	Toll-like receptor
TNF	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptor superfamily
TNX	Tenascin
Treg	T regulatory cell

3. ABSTRACT

Background: Coronary artey disease (CAD) is an inflammatory disease, in which several novel risk factors are believed to be involved, e.g., chronic infections, inflammation, genetics, etc. A majority of genes in the major histocompatibility complex (MHC) region are involved in the regulation of infection, inflammation, and immune responses.

Aims: We aimed to examine the roles of MHC genes on CAD, *Chlamydia pneumoniae* (*C. pneumoniae*), periodontitis, and periodontal pathogens.

Methods: Two separate patient cohorts and controls were enrolled. The first cohort consisted of heart transplant (HTx) recipients (n = 276), who were divided into 3 subgroups based on the severity of coronary atherosclerosis in the explanted heart; and the second cohort of patients with acute coronary syndrome (ACS, n = 148), and age- and sex-matched healthy controls (n = 74). Patients with ACS were monitored for subsequent ischemic cardiovascular events (composite end-points of death, myocardial infarction, recurrent unstable angina, or stroke). Forty-four of them met an end-point during the follow-up period (555.4 ± 21.2 days). MHC markers for HTx recipients (HLA-A, HLA-B, and HLA-DR), and ACS patients and controls [HLA-A, HLA-B, LTA+253(a/g), LTA+496(C/T), LTA+633(c/g), LTA+724(C/A), C4A, C4B, and HLA-DRB1] were typed and their haplotypes were inferred. Blood samples were taken at the time of hospitalization. Following parameters were measured from serum samples: complement C3, C4, *C. pneumoniae* markers (IgA, IgG and IC), and markers of periodontal pathogens (IgG and IgA antibodies to both *Aggregatibacter actinomycetemcomitans* [*A. actinomycetemcomitans*] and *Porphyromonas gingivalis* [*P. gingivalis*]). Based on the panoramic tomography taken during initial hospitalization, dentate patients with ACS were categorized into groups of non-periodontitis and periodontitis. The C3/C4 ratio was calculated from serum C3 and C4 concentrations.

Results: Study I showed that the HLA-DRB1*01 -related haplotype (HLA-A3− B35− DR1) emerged as a risk for CAD. HLA-DR1 alone associated with CAD, and CAD risk factors, e.g., diabetes mellitus, increased serum low density lipoprotein level, smoking, and C4B quantitative null allele (C4B*Q0). On the other hand, the HLA-B*07 and HLA-DRB1*15 -related haplotypes were found to be protective markers for CAD. Study II showed that HLA-B*35 and -related haplotypes were associated with elevated serum markers of *C. pneumoniae* infection. Among patients with HLA-B*35 and -related haplotypes, *C. pneumoniae* infection markers were more pronounced in smokers and in males. Study III showed that lymphotoxin alpha exonic variant (LTA+496C) strongly associated with panoramic tomography-defined periodontitis; whereas, increased occurrence of *P. gingivalis* in saliva and elevated serum antibodies of the pathogen were associated with HLA-DRB1*01 and patients with periodontitis. Study IV showed that the increased C3/C4 ratio was associated with recurrent cardiovascular end-points. Patients having both higher than the cut-off value (4.53)

and higher than the highest quartile of the C3/C4 ratio were associated with the end-points and had the worst survival rates. The relative increase in serum C3 protein level and decrease in C4 protein level could explain the changes in the C3/C4 ratio. The presence of C4 null alleles showed the increased level of C3/C4 ratio, and decreased level of serum C4 concentration.

Conclusions: The HLA-A3− B35− DR1 -related haplotypes are a risk for CAD, whereas the HLA-B*7 and HLA-DR*15 -related ones are protective. Elevated markers of *C. pneumoniae* were associated with HLA-B*35 and -related haplotypes. HLA-B*35 may be a link between chronic *C. pneumoniae* infection and CAD. LTA+496C was associated with periodontitis; whereas serum elevated antibodies of *P. gingivalis*, and increased occurrence of the pathogen in saliva were associated with HLA-DRB1*01. As a new cardiovascular risk factor, the increased C3/C4 ratio was associated with recurrent cardiovascular end-points, and C4 null alleles partially explained the increased C3/C4 ratio.

4. INTRODUCTION

Atherosclerosis is an immunologically-induced inflammatory disease (1). Coronary atherosclerosis is the major cause for coronary artery disease (CAD) (2), in which several novel risk factors are believed to be involved, e.g., infections, impaired host immune responses, genetics, etc. The major histocompatiblity complex (MHC) region contains several genes with essential functions in the susceptibility and protection against infections, but also in inflammation and immune responses (3). Polymorphic MHC genes are in linkage disequilibrium forming conserved haplotypes (4). Such haplotypes may be population-specific, and can be used to specify or evaluate different disease groups (5-8). On the other hand, CAD associates with several MHC genes, e.g., tumor necrosis factor (TNF), lymphotoxin alpha (LTA), and C4A and C4B quantitative null alleles (C4A*Q0 and C4B*Q0) (9, 10), but these genes have never been evaluated in combination with other human leukocyte antigen (HLA) genes.

Infections have important roles in the pathogenesis of atherosclerosis, and *Chlamydia pneumoniae* (*C. pneumoniae*) is one of the best-studied (11). During lifetime most likely all individuals get infected with *C. pneumoniae*, but many can resolve the infection, while some become chronically infected. Interferon gamma (IFN-γ) (12), antibiotics (13), and tobacco smoke (14) can induce the persistent form of *C. pneumoniae*. Host immunogenetic factors (15) may also contribute to the outcome of the infection. Another example of common bacterial infection is adult periodontitis. Periodontitis is characterized by chronic inflammation in the tooth-supporting tissues, which gradually destroys the attachment of teeth to alveolar bone, and eventually may lead to the loss of teeth. Periodontitis (16) as well as *C. pneumoniae* infection (11) are risk factors for cardiovascular disease. In addition to environmental risk factors involved in these diseases, host immune system, and genetics play important roles (15, 17-21).

In CAD, the complement system is activated (22, 23). More than 30 different plasma and cell surface proteins are activated in a cascade sequence. Activated complement system has an essential role in host defense against infections and in elimination of circulating immune complexes (22, 23). Activation of the complement system might need the optimum level of complement components. The presence of C4 null alleles (C4A*Q0 and C4B*Q0) (24-26) and the increased serum C3 concentration (27-29) are risks for CAD. In the presence of C4 quantitative null alleles, the level of C4 synthesis may not match the need, partially explaining the C4 specific deficiency (30, 31). The increased production of C3 and decreased production of C4 are also regulated by different inflammatory cytokines. Thus, the ratio of serum C3 and C4 concentrations can be affected by the degree of inflammation.

The aims of this study were to evaluate whether the human MHC region harbors genes that associate with CAD and its risk factors, e.g., *C. pneumoniae*, periodontitis, major periodontal pathogens, and whether C3 and C4 concentrations influence the recurrent cardiovascular events.

5. REVIEW OF THE LITERATURE

5.1. Coronary artery disease

CAD is the most common cause of death in industrialized countries, and its prevalence is rapidly increasing also in developing countries. In Finland alone, 12,000 people per year suffer from acute myocardial infarction (32, 33). The most common clinical manifestations of CAD are angina pectoris, myocardial infarction, heart failure, rhythm problems, and eventually death. The incidence of CAD increases with age. More than 80% of the patients dying from acute myocardial infarction are older than 65 years (34). Males suffer from CAD more often at younger age than females, but females may be sicker than males when they are treated for heart attack for the first time.

Atherosclerosis in coronary arteries is a major cause of CAD. Atherosclerosis is an organized, active, lifelong process that involves the elements of inflammation followed by repair in the arterial wall forming a plaque (35). At the beginning, the maturing or growing plaque usually does not compromise the lumen, but it can affect the vessel wall. Coronary angiograms taken during this stage may not show any evidence of CAD, because the coronary artery lumen has not decreased in calibre. With further growth of the plaque, the vessel lumen also becomes compromised and the blood flow through the vessel is impaired (Figure 1).

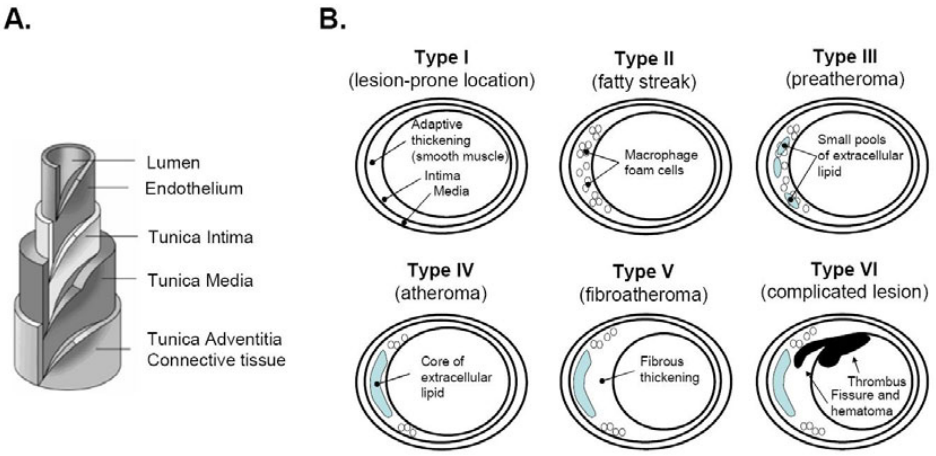


Figure 1. Quadrilaminar structure of a normal artery (A), and histological classification of atherosclerotic lesions (B). Type I and II lesions are referred to as early, type III as intermediate and type IV-VI as advanced lesions. Modified from: Stary HC et al (36).

The hemodynamic significance of a plaque varies depending on the length and morphology of the lesion. Over 50% decrease in the luminal diameter may limit blood flow during increased demand (e.g., in exercise), while a 90% stenosis may be needed to limit the flow in the resting state (37). Fissure or rupture in the margin of the plaque, or shoulder region, results in the exposure of highly thrombogenic collagen and lipid to circulation, with a resultant formation of intraluminal thrombus. Activated platelets mediate vasoconstriction and further thrombus propagation, abruptly compromising the coronary blood flow. The severity of plaque

rupture and extent of thrombosis are clinically reflected in the spectrum of acute coronary syndrome (ACS) (37).

Clinical spectrum

The clinical spectrum of CAD ranges from silent ischemia (asymptomatic) to chronic stable angina, unstable angina, acute myocardial infarction, and sudden cardiac death. Patients with CAD are usually divided into two major groups, those with stable angina and those with ACS. Stable anginal pain is induced by physical activity or emotional stress, and relieved by rest. Stable angina usually shows up to 70% stenosis resulting from an atherosclerotic plaque in one or more coronary arteries. ACS shows the fissured plaque with platelet and fibrin thrombus contributing to stenosis. Patients with ACS can be divided into three major groups: unstable angina pectoris (not associated with heart muscle damage), and two forms of acute myocardial infarction where the heart muscle is damaged. These types are named according to their appearance in an electrocardiogram as ST-segment elevation myocardial infarction, and ST-segment depression myocardial infarction. The pathophysiology of stable angina differs from ACS, and probably the severity of inflammation is stronger in ACS than in stable angina (38). Patients with CAD reflect signs of inflammation, e.g., serum elevated levels of C-reactive protein, leukocytes, and fibrinogens (1).

Risk factors

Risk factors for CAD are divided into non-modifiable and modifiable risk factors. Non-modifiable risk factors include male gender, advancing age, and a family history of CAD. Modifiable risk factors include tobacco smoking, high blood pressure, high blood cholesterol, diabetes, overweight, and physical inactivity. Modifiable risk factors are strongly influenced by environmental factors, e.g., people with poor socioeconomical status are more prone to these risk factors. Smoking increases the risk of premature death to 1.63 fold (Cox proportional hazard ratio) in middle-aged men (39). High blood pressure ascribes for 5.0% of total mortality in middle-income countries; similarly, tobacco for 4.0%, high cholesterol for 2.1%, and obesity for 2.7% (40). On the other hand, physical activity has beneficial effects on high density lipoprotein levels in elderly men (41). However, about 10% to 50% of patients with CAD lack these environmental and traditional risk factors (42), and these factors are also influenced by other factors, e.g., genetics. Several novel risk factors (e.g., homocystein, abnormal blood coagulation, chronic infections, inflammatory markers, genetics, etc.) are also gradually emerging, but the evidence of their independent causal roles is poorly understood.

Pathophysiology

CAD is known as a complex multifactorial disease, and attempts to explain it have been evolving over the time (Figure 2).

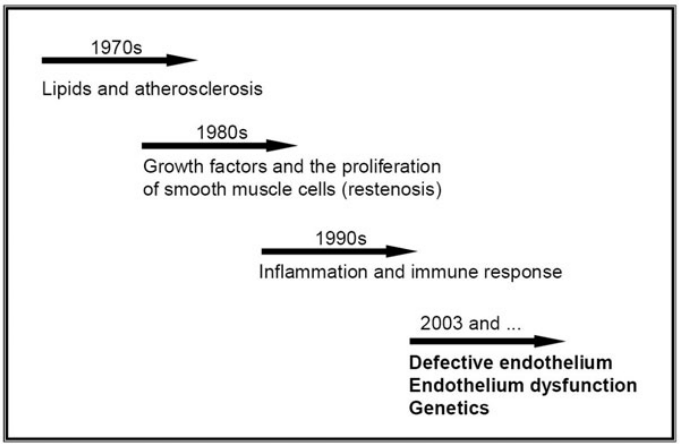


Figure 2. Views of the pathophysiology of coronary disease. The lipid hypothesis was proposed by the German pathologist Rudolph Virchow in 1856 who suggested that blood lipid accumulation in arterial walls causes atherosclerosis (43). Modified from: Wang Q (19).

"Response-to-injury" hypothesis is one of the modern hypotheses (35, 44). Endothelial dysfunction is an important factor, which leads to the initiation of vascular inflammation. It induces several genes (Figure 3) initiating the production of several immune components, which are prerequisites for inflammatory reactions. By now it is well-accepted that atherosclerosis is an inflammatory disease (1).

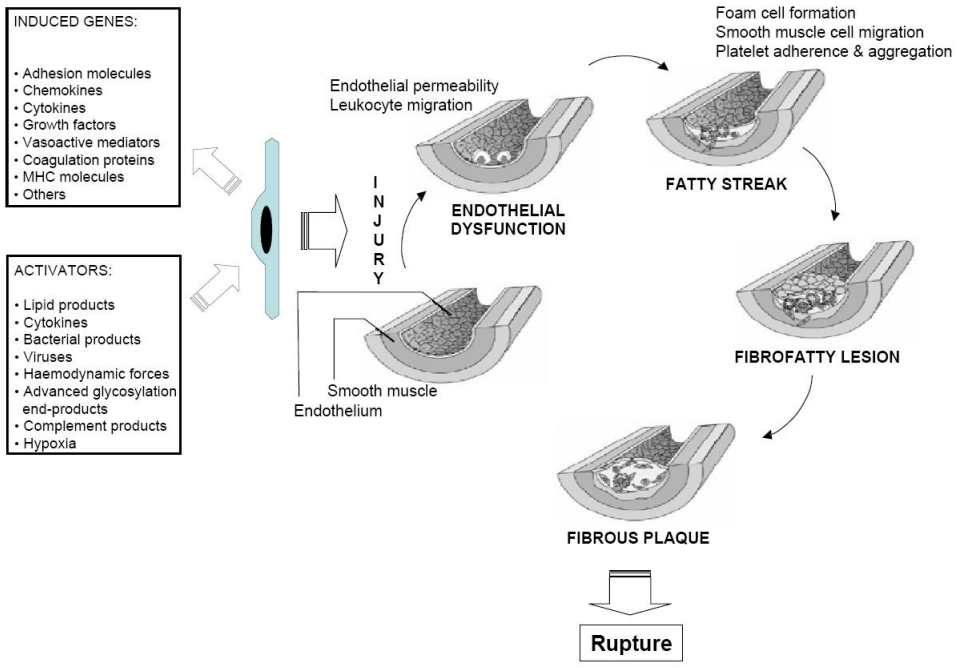


Figure 3. Endothelial dysfunction and response-to-injury hypothesis. MHC = Major histocompatibility complex. Based on: Ross R (35).

Family history has long been an important predictor for familiar aggregation with inheritability estimated to be even greater than 50% (45, 46). Thus, genetics, including immune genes, may be involved in the pathogenesis of CAD, probably modulating the inflammatory reactions.

5.2. Coronary artery disease as an inflammatory disease

Sequences of complex inflammatory cascades in atherosclerosis are described. The excess of low density lipoprotein (LDL) diffuses tunica intima, where LDL undergoes oxydative and enzymatic modifications. This leads the endothelial cells to express adhesion molecules (e.g., vascular cellular adhesion molecule-1) which bind to circulating monocytes and T cells via their very large antigen 4. This binding initiates leukocyte migration into the intimal layer in response to chemoattractants (e.g., chemokines, Figure 4).

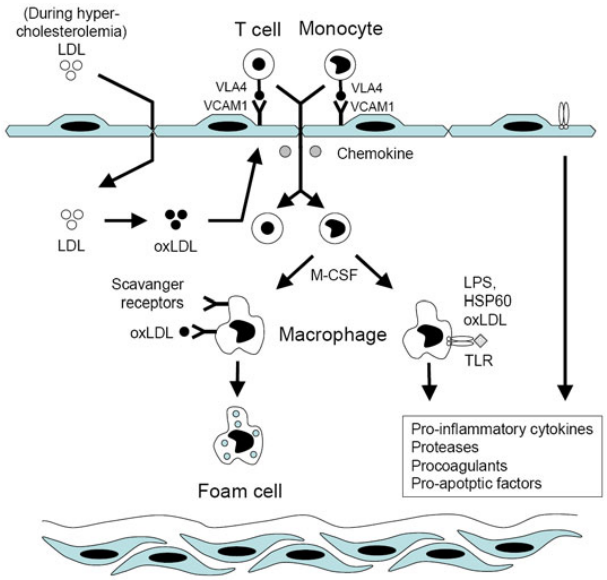


Figure 4. Migration of monocytes and T cells into intima, and formation of foam cells and activation of macrophages. See Appendix for abbreviations.

The macrophage colony-stimulating factor differentiates monocytes into macrophages, and macrophages up-regulate the pattern recognition receptors (e.g., scavenger receptors, toll-like receptors [TLRs], etc., Table 1). The immune response followed by the pattern recognition receptors belongs to the innate immune system.

Table 1. Pattern recognition molecules and their ligands.

Pattern recognition molecules	Patterns
Complement	Microbial cell wall components
Mannose binding lectin	Mannose-containing microbial carbohydrates
C-reactive proteins	Phosphatidylcholine (microbial membranes)
LPS-binding protein	Bacterial LPS
TLR2	Cell wall components of gram positive bacteria, LPS; yeast cell wall components
TLR3	Double stranded RNA (replication of many viruses)
TLR 4	LPS
TLR5	Flagellin (flagella of gram positive and gram negative bacteria)
TLR9	
Scavenger receptors (many)	Many targets; gram positive and negative bacteria, apoptotic host cells

LPS = Lipopolysaccharide, TLR = Toll-like receptor.

The scavenger receptors endocytose a wide range of molecules, e.g., bacterial particles, oxidized LDL (oxLDL), and apoptotic cell fragments. Intracelullarly, these molecules are degraded into peptides. The degraded peptides are presented to the T cells via MHC molecules. This step links innate immunity to adaptive immunity (Figure 5). If macrophages cannot mobilize the endocytosed oxLDL, the oxLDL accumulates in the cytosole leading to the formation of foam cells (Figure 4).

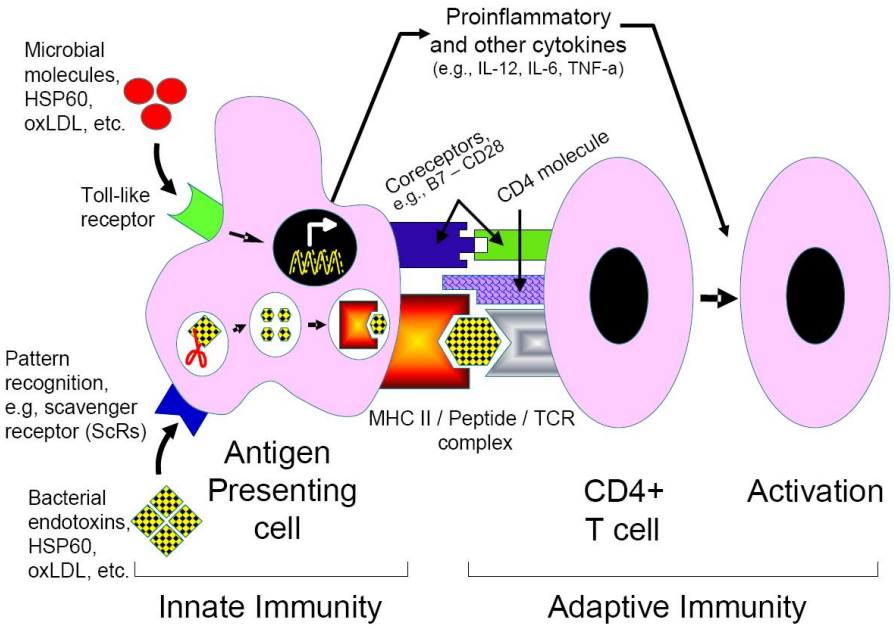


Figure 5. Major histocompatibility complex molecules linking innate immunity and adaptive immunity. CD4 = CD4 molecule, HSP60 = Heat shock 60 kDa protein, MHC = Major histocompatibility complex, oxLDL = Oxidized low density lipoprotein.

Among the TLR receptors, TLR4 is known to affect the initiation and progression of atherosclerosis (47). Both endogenous and exogenous ligands can activate TLR4. The activated TLR4 leads to activation of the nuclear factor κB or interferon regulatory factor 3-responsive genes (47) (Figure 6).

The TLRs do not endocytose, but ligate a wide range of molecules (e.g., pathogen-associated molecules, stress proteins, e.g., heat shock 60 kDa protein, deoxyribonucleic acid motifs, and oxLDL). This ligation induces the nuclear factor-κB and mitogen-activated protein kinase activator protein 1 signalling pathways. Activation of these pathways induces the expression of several molecules (e.g., proinflammatory cytokines, proteases, e.g., matrix metalloproteinases, and vasoactive molecules, such as nitric oxide, endothelins, several eicosanoids, and reactive oxygen species) leading to the activation of macrophages and T cells (Figure 7). The expressed proteases degrade the matrix components, leading to destabilization of plaques and an increased risk for plaque rupture (48).

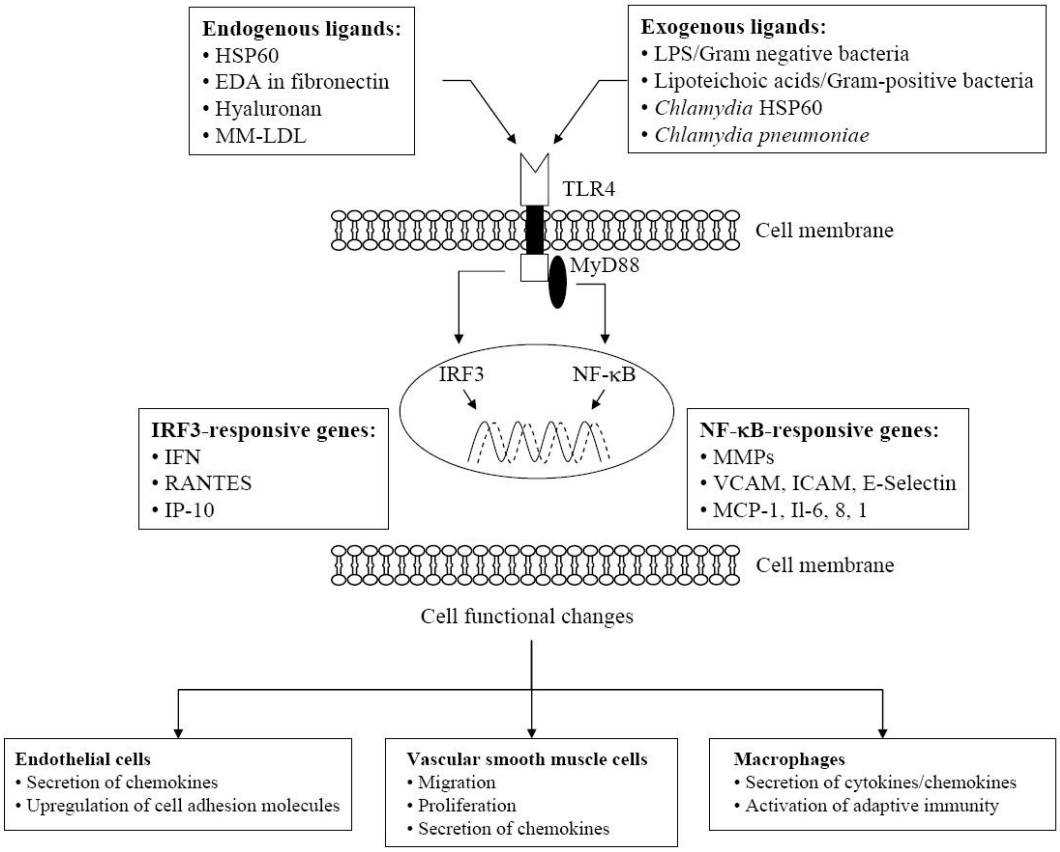


Figure 6. Toll-like receptor type 4 signaling pathway and its relation with atherosclerosis. See Appendix for abbreviations. Modified from: Li H et al (47).

Roles of T cells

T cells and MHC class II-expressing cells are found in the shoulder regions of atherosclerotic plaques (49, 50), and it has been shown that the antigen presentation restricted to class II molecules initiates the activation of T helper cells. Based on the expressed mediators, the Th cells can be divided into three major groups: Th1, Th2, and Treg (T regulatory) cells. Usually, the Th1 cells induce the cell-mediated immune response, while the Th2 cells induce the humoral immune response. The Treg cells, also known as suppressor T cells, suppress the activation of the immune system. See also Section 5.4.3.2.

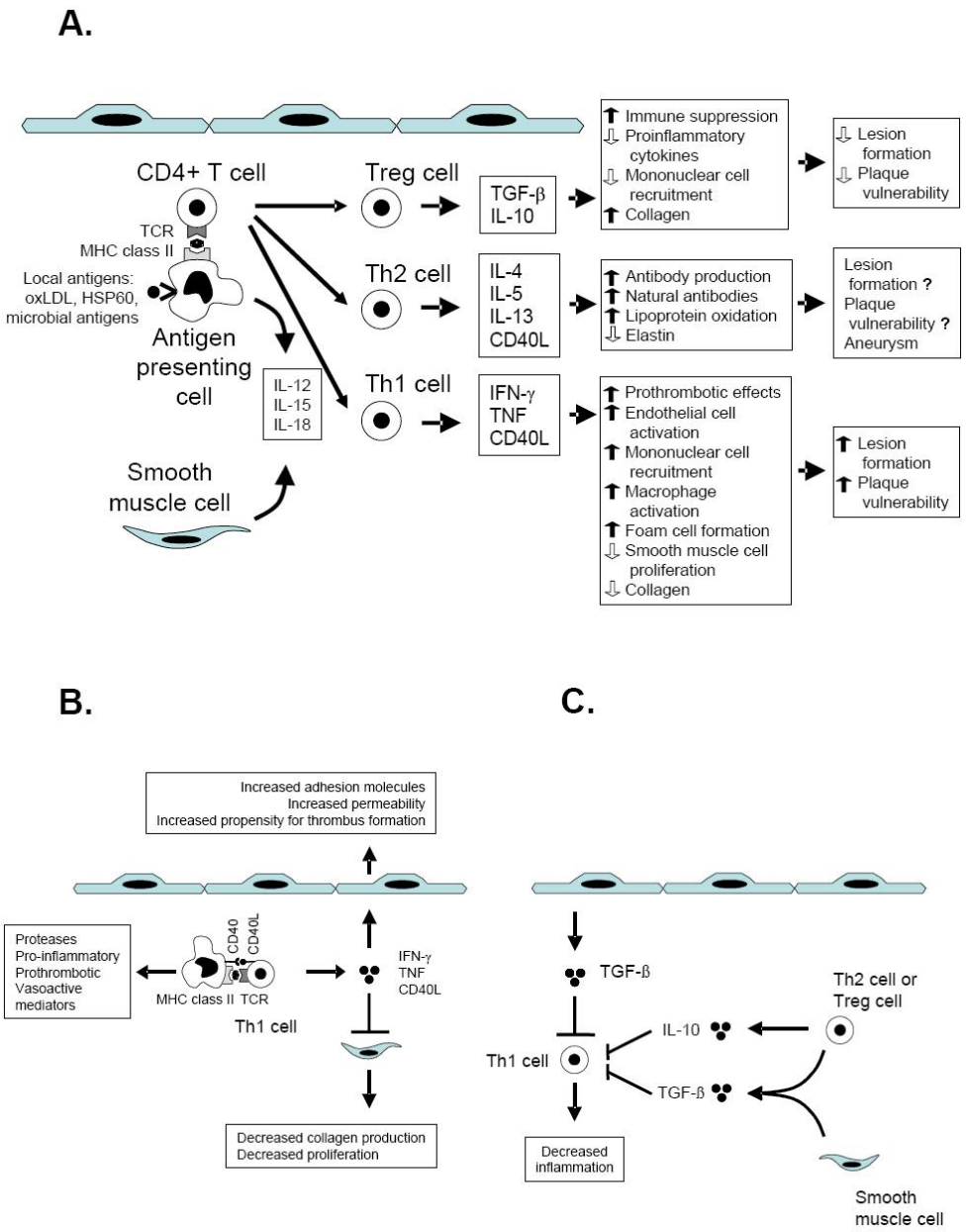


Figure 7. T cell responses in atheroclerosis (A), activation of Th1 cell (B), and Th2 and Treg cell (C). See Appendix for abbreviations.

Th1 cells produce mainly IFN-γ, TNF, and CD40L (Figure 7). IFN-γ activates macrophages to produce nitric oxide, proinflammatory cytokines, and vasoactive and prothrombotic mediators, but they also inhibit the proliferation of endothelial and smooth muscle cells, and decrease collagen production (51-53). TNF causes vascular inflammation following the nuclear factor κB pathway, and induces accumulation of liporoteins, thrombosis, and production of reactive oxygen and nitrogen species, proteolytic enzymes and prothrombotic tissue factors. CD40 and CD40L molecules trigger an inflammatory response similar to TNF (54).

Th2 cells produce mainly IL-4, IL-5, IL-13, and CD40L (Figure 7). IL-4 and IL-13 are the major stimuli for antibody production, while IL-5 promotes eosinophilic inflammation, and antibody secretion. IL-4 induces

matrix metalloproteinase type 1 (by macrophage) production, the activation of which is known to proteolyse collagen fibrils (55).

Treg cells are considered to maintain the self-tolerance and control of autoimmunity. Treg cells produce mainly IL-10, and TGF-β (Figure 7). IL-10 has immunosuppressive effects and it inhibits the secretion of proinflammatory cytokines [e.g., IFN-γ (56)], reduces the pro-matrix metalloproteinase activity (57, 58), and induces the production of TGF-β (and vice versa) (57, 59). TGF-β has also imunosuppressive effects (60, 61). The mechanism of TGF-β is not fully understood; however, it is known to be atheroprotective (62, 63), probably it modulates or reduces T cell activity, and helps in stabilizing the plaques by promoting collagen production (64, 65).

Briefly, the formation, development, and rupture of an atherosclerotic lesion seem to depend on the balance of Th1, Th2, and Treg cells responses. If the balance leans towards the Th1 responses, it may result in the growth and rupture of an atherosclerotic lesion (66). Similarly, Th2 response may result in aneurysm (67); whereas, Treg response may inhibit lesion growth (61, 68).

5.3. Coronary artery disease as an infectious disease

The reason for inflammation in the vessel wall during the formation or development of atherosclerosis is not fully understood; however, the most frequently suspected candidate is oxLDL (1). Other candidates include infections.

In 1979, an avian herpes virus was shown to induce arterial changes that resembled human atherosclerosis in an experimental infection of germ-free chickens (69). This initiated a new vision that infection may cause ischemic heart disease. Later, cardiovascular diseases have been associated with either viral (e.g., cytomegalovirus, herpes simplex virus type 1 and 2, influenza, enteroviruses, hepatitis A, etc.) or bacterial (e.g., *C. pneumoniae*, *Helicobacter pylori*, periodontal pathogens, etc.) infections (11, 70). Furthermore, infections seldom appear alone. An “infectious burden” hypothesis has also been proposed, suggesting the contribution of multiple infections (71), which seems to be a stronger determinant of coronary heart disease risk than any single infection (72, 73).

Numerous studies link infections to atherosclerosis. It remains unclear whether these pathogens are causal factors. Pathogens link to vascular diseases either directly or indirectly (Figure 8). A direct link requires the actual presence of pathogens or toxins that interact with the vessel wall. An indirect link, absence of actual presence of pathogens or toxins, results the changes in homeostasis or the production of circulating soluble mediators that open several pathways leading to vascular inflammation.

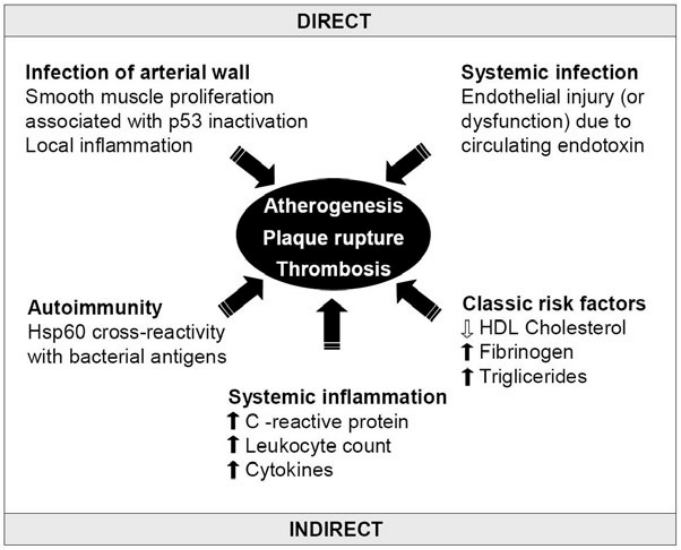


Figure 8. Mechanisms linking infections to vascular disease. HDL = High density lipoprotein cholesterol, Hsp60 = Heat shock 60 kDa protein, P53 = Protein P53. Modified from: Danesh J et al (74).

Local infection may cause subclinical bacteremia or viremia. These circulating pathogens or other soluble components may cause atherosclerosis-related vascular inflammation. The role of pathogens, transferring from normal vessel wall to the inflamed one, is complex; however, some mechanisms have been proposed (Figure 9):

1. Pathogens or pathogen-specific toxins (e.g., endotoxins) can directly infect and activate the endothelial cells. This may alter the endothelial integrity, smooth muscle cell proliferation, and cytokine expression (*direct invasion*).
2. Locally-produced soluble mediators of inflammation enter the blood stream, and interact with the endothelial cells (*immunological sounding*).
3. Circulating infected phagocytes interact with endothelial cells. The localization of pathogen-infected phagocytes is initiated by the chemotactic gradiant (e.g., IL-8, macrophage chemotactic protein-1), and cellular adhesion is initiated by adhesion molecules (*pathogen trafficking*).
4. Pathogen molecules, which are homologous to host molecule (e.g., heat shock protein molecules) can initiate an autoimmune reaction. This may lead to the unwanted immune responses against self tissues that may cause vascular endothelial cell damage (*molecular mimicry*).

It seems that pathogens are equipped with several sophisticated attacking or invading mechanisms that cause atherosclerosis-related vascular inflammation. As pathogens evolve with their attacking or invading mechanisms, the host defense system attempts to defend them. The host defense system must evolve parallel to pathogens. Thus, the host-pathogen interaction may have an important role also in atherosclerosis (see Section 5.3.2 for details).

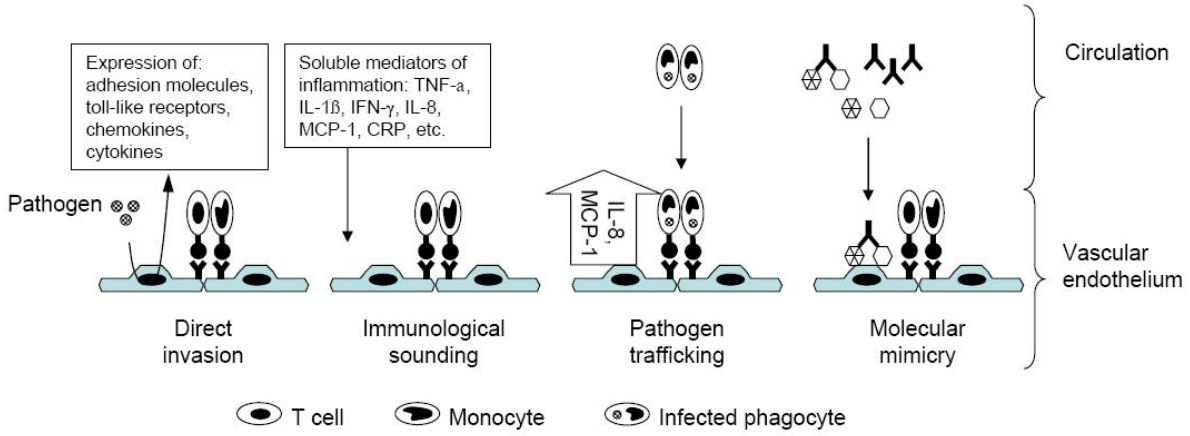


Figure 9. Four mechanisms that explain the interactions between infections and vascular endothelial cells which results vascular inflammation. Molecules indicated with diamond shapes depict bacterial and human heat shock proteins. CRP directly exhibits proinflammatory actions on vascular cells such as activation of classical complement pathway, induction of adhesion molecules, and inhibition of nitric oxide production (75-77). CRP = C-reactive protein, IFN = Interferon, IL = Interleukin, MCP-1 = Monocytyte chemoattractant protein 1, TNF = Tumor necrosis factor.

5.3.1. Chlamydia pneumoniae infection

C. pneumoniae is an obligatory intracellular gram-negative pathogen. It causes respiratory infections (e.g., pneumoniae, bronchitis, and sinusitis), but is also associated with certain chronic conditions of public health interest, such as atherosclerosis and cardiovascular diseases. Since two decades, its association with CAD has been described, showing the elevated *C. pneumoniae* IgA, IgG and IC levels in patients with CAD (78-81). Several studies have confirmed these findings (11). However, no clear explanations are available whether these antibodies are only a serological scar of the infection, or markers of chronic infection having a causal role in CAD.

Evidence supporting the *C. pneumoniae* infection in CAD

C. pneumoniae has strong evidence as a candidate pathogen for atherosclerosis. This can be explained by the following:

1. Several seroepidemiological studies have shown the association of chronic sublinical infection of *C. pneumoniae* with cardiovascular disease; however, some studies challenge it (78, 79, 82-85).
2. Electron microscopy, immunohistochemistry, and polymerase chain reaction studies show the presence of *C. pneumoniae* in atherosclerotic lesions (86, 87).
3. *C. pneumoniae* infection can modulate the function of atheroma-associated monocyte / macrophage in *in vitro* studies (88).
4. Animal studies show that *C. pneumoniae* can promote the initiation or progression of atherosclerosis, and antibiotic treatment can prevent it (89, 90).

5. Randomized antibiotic trials directed towards *C. pneumoniae* have failed to decrease ischemic cardiac events (Table 2); one can, however, argue that these unsuccessfull trials do not exclude the *C. pneumoniae* theory (87). It can also be assumed that these trials may not exclude a possibility of the combination with other agents, e.g., immunosuppressive agents.

Bacteriology

Chlamydiae follow a biphasic developmental cycle (91) (Figure 10). The elementary body is the infective and invasive phase of this cycle. It can survive extracellularly. The elementary body enters to eukaryotic cells through a phagosome, and reorganizes into a metabolically active form, a reticulate body, which is a non-infective form. The reticulate bodies survive only inside the intracellular vesicle (92), and mature into a new generation of elementary bodies, which infect other cells after releasing.

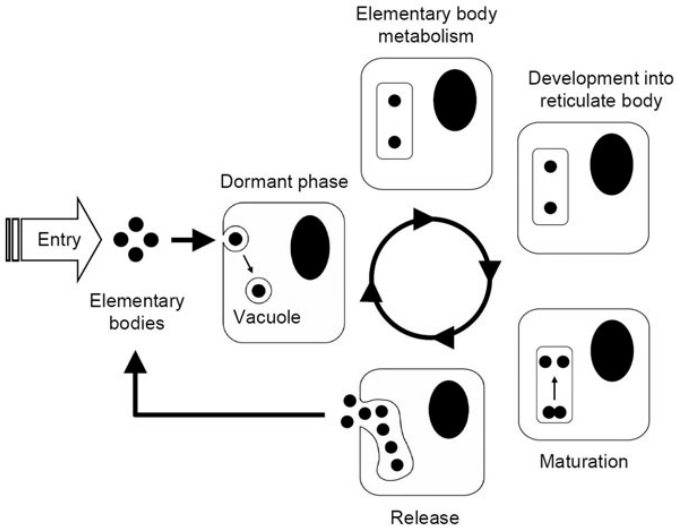


Figure 10. The biphasic developmental cycle of *Chlamydia pneumoniae*.

Antibodies (IgA, IgG and IC) to *C. pnemunomiae* are the most frequently used markers in seroepidemiological studies. The recommended positivity limits in microimmunofluorescence with TWAR antigen are 1/10 for IgA, 1/32 for IgG, and 1/2 for IC (78, 81); however, the 1/10 for IgA and 1/32 for IgG do not always show the presence of bacteria. Opinions vary that the first two can only be used as evidence of past exposure, and the presence of IC is a direct evidence only for the presence of chlamydial antigens; thus, not providing evidence of continuous infections.

Pathogenic mechanism by which *C. pneumoniae* could affect the development of atherosclerosis and coronary heart disease

C. pneumoniae infects the lower respiratory tract. There is evidence suggesting that infected leukocytes (e.g., CD3 T lymphocytes (93) and monocytes (94)) disseminate to other tissues (pathogen trafficking; Figure 9). Several studies describe the role of *chlamydial* components, interacting with the host, and resulting in the

formation and development of atherosclerosis. These components include, e.g., *Chlamydial* heat shock proteins, and lipopolysaccharides (Figure 11). *Chlamydial* heat shock proteins (e.g., 60 kDa protein) have high sequence homology with their human counterparts; thereby they can initiate autoimmune reactions (11, 95) (molecular mimicry; Figure 9). *Chlamydial* heat shock 60 kDa protein takes also part in the formation of oxLDL (96), and the production of proinflammatory mediators by macrophages, endothelium, and smooth muscle cells (97, 98). *Chlamydial* lipopolysaccharide induces macrophages to secret atherogenic components, e.g., TNF-α, and matrix metalloproteinases (88), and it can also elicit the formation of foam cells. Furthermore, *C. pneumoniae* can infect and multiply within macrophages and endothelial cells (99), and these endothelial cells can induce the proliferation of smooth muscle cells (100). *C. pneumoniae* can also infect the smooth muscle cells that elicit the secretion of cytokines (101), e.g., IL-6. Infected macrophages and endothelial cells can express leukocyte adhesion molecules, and proinflammatory cytokines (e.g., IL-1, IL-6, and TNF-α). Via these adhesion molecules, circulating leukocytes adhere to the infected endothelial cell, which enhances the migration of leukocytes and intimal inflammation (102).

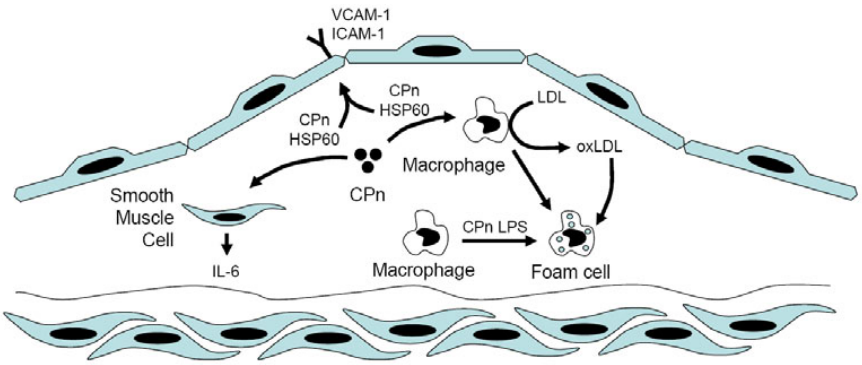


Figure 11. Effects of *Chlamydia pneumoniae* on atheroma. See Appendix for abbreviations. Modified from: O'Connor S et al (103).

C. pneumoniae secrets the protease-like activity factor (CPAF) into the host cytoplasm, where it degrades the host transcription factor (e.g., regulatory factor X 5) (see Section 5.4.3.3 for details). The regulatory factor X 5 is crucial for the MHC gene activation (104). This is in agreement with previous reports that *Chlamydia trachomatis* decreases IFN-γ induced expression of MHC class II molecules including HLA-DR in the infected cell (105).

C. pneumoniae induces complex immune responses in atherosclerotic plaques (106). *Chlamydiae* are able to elicit a variety of cell-mediated and humoral immune responses (107); however, the interplay between humoral and cell-mediated immune responses in terms of protection or adverse sequela of *chlamydial* infection is not thoroughly studied.

Attempts to eradicate *C. pneumoniae* in CAD

Seeking a reduction in the cardiac events, several antibiotic clinical trials have been launched; some preliminary trials gave promising results; but overall no benefits were found (Table 2). For these unsuccessful trials, several explanations were offered, and periodontitis is one of them. The beneficial effect of the antibiotic might be limited to individuals who do not have periodontitis (108, 109).

Thus, it is still under debate whether *C. pneumoniae* is directly involved in the pathogenesis of CAD, since biological mechanisms are plausible. Regardless of the ongoing debate, a vaccine to prevent *C. pneumoniae* infection would add a new dimension to the prevention of coronary heart disease. Accordingly, attempts aiming at *Chlamydial* major outer membarane protein (MOMP), and outer membrane protein 2 (Omp2) are proceeding (110).

Table 2. Effect of antibiotic treatment on mortality, myocardial infarction and acute coronary syndrome.

Studies	Intervention	Population (N)		Patients	Follow-up	Mortality[1]	MI[1]	ACS[1]
		Antibiotics	Placebo			OR (95% CI)	OR (95% CI)	OR (95% CI)
Gupta et al (111)	Azithromycin	40	20	Stable CAD	18 months	0.49 (0.03-8.22)	—	0.60 (0.16-2.30)
ACADEMIC (112)	Azithromycin	150	152	Stable CAD	2 years	1.28 (0.34-4.85)	0.67 (0.18-2.41)	0.93 (0.41-2.11)
ROXIS (113)	Roxithromycin	102	100	ACS	6 months	0.38 (0.07-2.01)	0.19 (0.01-4.05)	0.63 (0.22-1.85)
CLARIFY (114)	Clarthromycin	74	74	ACS	555 days	4.17 (0.46-38.24)	0.27 (0.09-0.76)	0.31 (0.12-0.75)
Leowattana et al (115)	Roxithromycin	43	41	ACS	3 months	0.95 (0.06-15.75)	0.60 (0.16-2.30)	0.21 (0.02-1.27)
STAMINA (116)	Azithromycin	111	107	ACS	1 year	0.96 (0.27-3.42)	—	0.64 (0.34-1.19)
WIZARD (117)	Azithromycin	3866	3856	Stable CAD	14 months	0.93 (0.75-1.14)	0.94 (0.75-1.19)	0.97 (0.821-1.16)
ANTIBIO (118)	Roxithromycin	431	437	ACS	1 year	1.10 (0.63-1.91)	0.88 (0.48-1.61)	1.21 (0.87-1.68)
AZACS (119)	Azithromycin	716	723	ACS	6 months	0.79 (0.46-1.39)	0.78 (0.41-1.47)	—
ACES (120)	Azithromycin	2004	2008	Stable CAD	4 years	1.08 (0.84-1.37)	1.04 (0.82-1.34)	1.10 (0.81-1.25)
PROVE-IT (121)	Gatifloxacin	2076	2086	ACS	24 months	1.30 (0.89-1.89)	0.89 (0.70-1.13)	0.93 (0.77-1.13)
CLARICOR (122)	Clarthromycin	2172	2200	ACS	3 years	1.19 (0.95-1.49)	—	—
Total		**11785**	**11804**			**1.06 (0.94-1.19)**	**0.92 (0.81-1.04)**	**0.91 (0.76-1.07)**

[1] OR less than 1 indicates the trial favors antibiotic. Modified from: Andraws R et al (123), and Jespersen CM et al (122).

5.3.2. Periodontal infections

The periodontium is comprised of the gingiva, cementum, alveolar bone, and periodontal ligaments. Periodontitis is a common bacterial infection in adults, and is characterized by chronic inflammation in tooth-supporting tissues or periodontium that gradually destroys the attachment of teeth to alveolar bone, and eventually may lead to loss of teeth. Periodontitis is associated with several subgingival bacteriae. Socransky SS et al (124) have classified them with five major complexes, as the first (e.g., *Porphyromonas gingivalis*), the second (e.g., *Prevotella intermedia*), the third (e.g., *Streptococcus sanguis*), the fourth (e.g., *Compylobacter concisus*), and the fifth (e.g., *Veilonella parvula*) complex. The first complex relates strikingly to clinical signs of periodontal disease.

Pathophysiology

Periodontal disease is a multifactorial disease, the exact etiology of which remains unclear. Hypotheses explaining periodontitis have been evolving over the time (Figure 12).

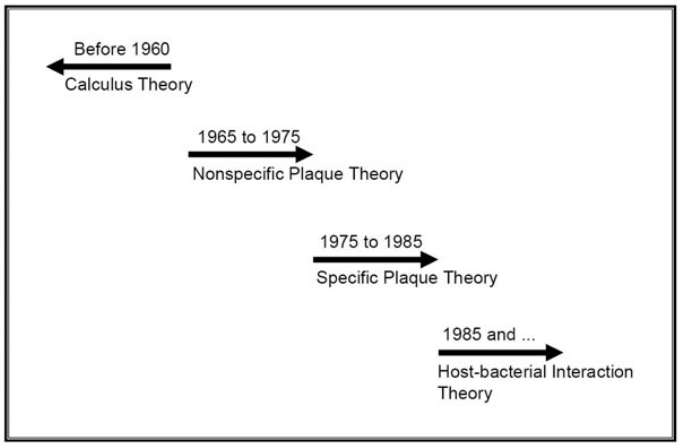


Figure 12. Hypotheses of the pathophysiology of periodontitis. Calculus acts as a mechanical irritant, the sole cause of disease (125) (*Calculus theory*). Bacterial plaque causes gingivitis and, in large enough quantities, could cause gingivitis to progress to periodontitis; all plaques are the same; too much plaque causes the disease (125) (*Nonspecific plaque theory*). Microbial paque becomes differentiated – some bacterial species are indentified as pathogenic and especially virulent in evading host defences; research centered on the composition instead of the quantity of plaque (125) (*Specific plaque theory*). Interaction of the host (patient's immune response) with pathogenic bacteriae, that determines whether periodontal disease is initiated or whether the disease progresses; recognition of certain risk factors that make certain patients more susceptible to disease (125) (*Host-bacterial interaction theory*).

Based on the modern hypothesis, periodontitis is characterized as a result of complex interactions between the host and the pathogen with a strong immunological background showing the nature of complex multifactorial disease (Figure 13).

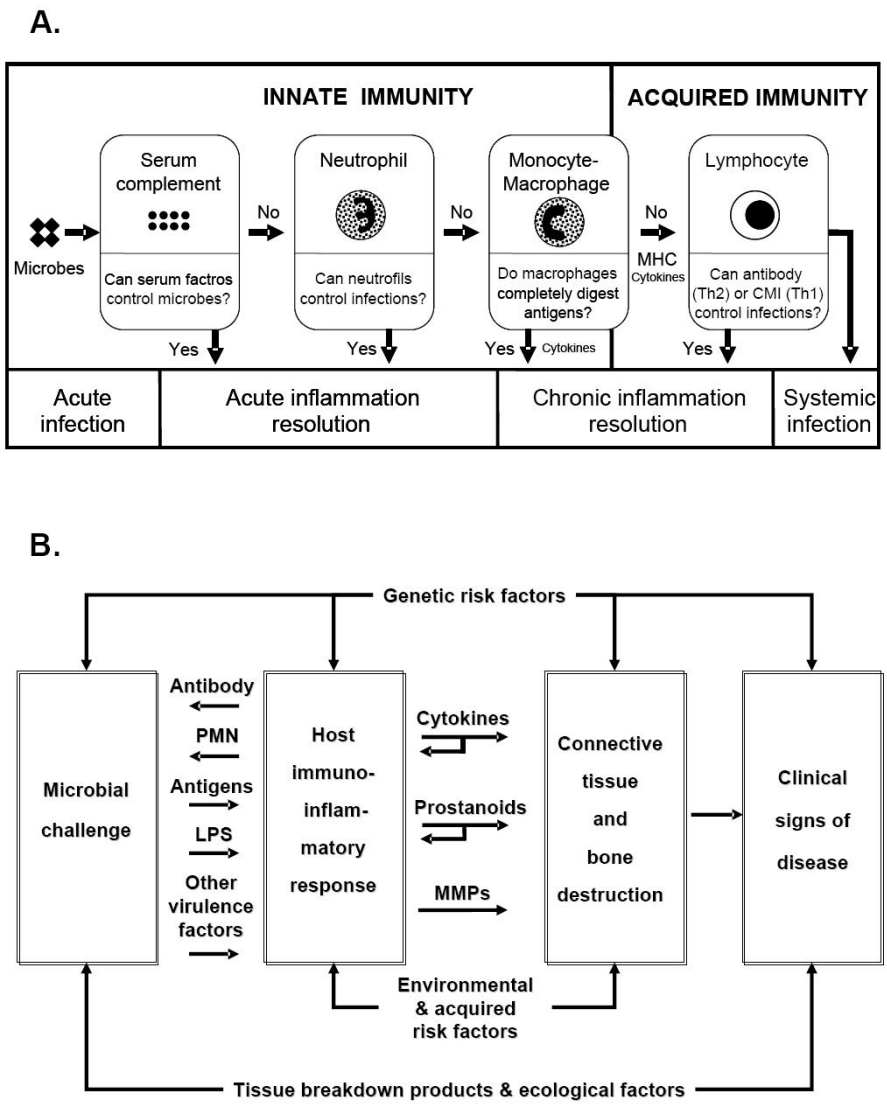


Figure 13. Host-defence against local and systemic infections of periodontal origin (A), and pathways linking to periodontitis (B). CMI = Cell mediated immune response, LPS = Lipopolysaccharide, MMP = Matrix metalloproteinase, PMN = Polymorphonuclear leukocyte, Th = T helper cell. Modified from: Miyasaki KT (126), and Page RC et al (127).

Inflammation in periodontium leading to periodontitis

Subgingival bacteriae form a mibrobial biofilm on the gingival sulcus, forming a dental plaque (Figure 14). A dental plaque is a host-associated biofilm. A dental pellicle coats on the surface of the tooth, where the bacteria start colonizing, continuing with secondary colonization and as a result, maturation of plaque occurs. Locally, the pathogens or toxins from the gingival sulcus interact with the immune cells, underlying periodontal and connective tissues. This interaction stimulates immune cells inducing the complex inflammatory cascade (128) (Figure 14). The macrophages producing several inflammatory mediators (e.g., IL-1, TNF) (129) induce complex inflammatory responses, including vascular changes with increased permeability leading to the increased exudation. Neutrophils are attracted to the site of inflammation by the cytokines expressed by macrophages. Neutrophils are capable to phagocyte micro-organisms. This phagocytic event results in the formation of reactive oxygen species, hydrolytic enzymes, and hypoclorous

acid. Local inflammatory cells release wide a range of enzymes and other destructive mediators, and they induce an endogenous host pathway to degrade the connective tissue, basement membrane as well as the oral epithelium. Furthermore, the activation of osteoclasts degrades the alveolar bone. The progression to periodontitis is regarded to be the conversion of junctional epithelium to the pocket epithelium, and the clinical sign is the formation of periodontal pocket.

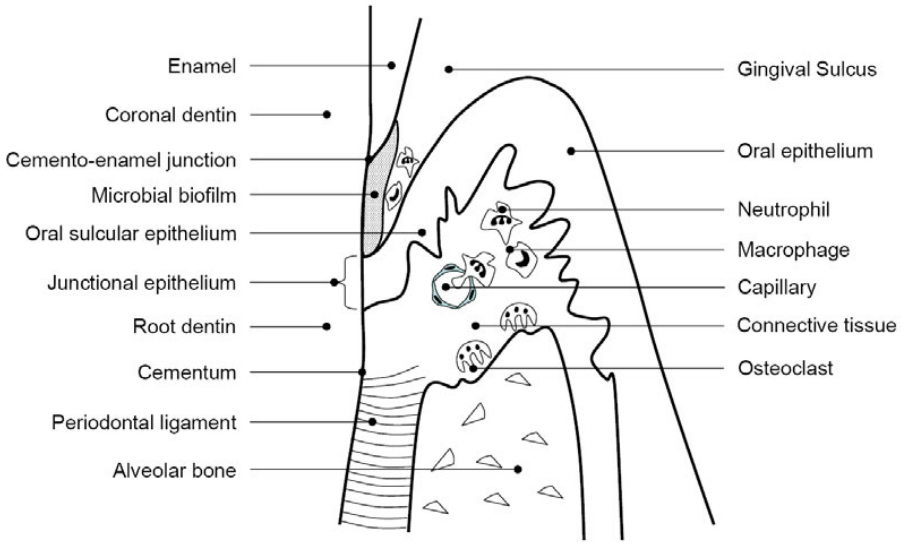


Figure 14. Microbial biofilm-induced local immune response (Bucco-lingual section).

Periodontitis and cardiovascular disease

Simonka M et al (130) and Mattila KJ et al (131) have introduced the association between dental infections and cardiovascular diseases. Since then, several studies have been performed. By now, there is evidence suggesting that the periodontal disease is associated positively but modestly with the increased risk for cardiovascular disease (16). A meta-analysis of prospective and retrospective follow-up studies has shown that periodontal disease may increase the risk of cardiovascular disease by approximately 20%. Similary, the risk ratio between periodontal disease and stroke is even stronger, varying from 2.85 to 1.74 (132).

The mechanisms how periodontal diseases cause heart diseases remain unclear. Some risk factors might partially explain the association between periodontitis and cardiovascular disease. Both diseases follow similar risk factors, e.g., male gender (133), increasing age (134), smoking habit (135), diabetes mellitus (136), and hyperlipidemia (137). Other biomarkers of cardiovascular disease can also be seen in periodontitis, e.g., von Willebrand factor antigen (138), fibrinogen (139), and C-reactive protein (136). Furthermore, both diseases follow the infection etiology, e.g., *P. gingivalis* is shown to be a risk for both diseases.

Evidence supporting the link between dental infection and CAD

1. Several seroepidemiological studies show that oral infection is a risk for CAD (16, 132).

2. Studies show that periodontal pathogen DNA (e.g., *P. gingivalis*, and *A. actinomycetemcomitans*) are found in atherosclerosis lesions (128, 140, 141). About 44% of the lesions had positives with one or more periodontal pathogens (128).
3. Both diseases show strong evidence of pathogens triggering specific inflammatory components (142). Oral pathogens initiate or accelerate atherosclerosis: e.g., *P. gingivalis* follows several mechanisms such as direct invasion, immunological sounding, pathogen trafficking, and molecular mimicry (Figure 15). Serum markers of major periodontal pathogens, *P. gingivalis*, and *A. actinomycetemcomitans*, have been shown to associate with the increased risk of stroke and coronary disease (143-147).
4. Well-established risk factors for CAD (e.g., diabetes mellitus, smoking, advancing age, and male gender) are also risks for periodontal disease. Similarly, the pathobiology of one disease gives evidence of another, e.g., cholesterol and lipid metabolism are the primary risk factors for CAD, and they have been shown to be at abnormal levels also in periodontal disease (148).
5. Based on the disease pathogenesis, several sets of candidate genes are available, e.g., genes related to connective tissue, bone, immune response, inflammation, lipid metabolism, and coagulation factors. For biological intersections between both diseases, genes involved in the inflammatory processes appear to be a viable first target for investigation. However, at the present, limited data are available to support any specific candidate gene as the explanation for observed associations between these two diseases (17, 18).
6. Periodontal treatment is effective in reducing the markers associated with cardiovascular disease, e.g., C-reactive protein and TNF-α; thus, it may decrease the risk of cardiovascular disease (149, 150). On the other hand, periodontitis is a failure-causing factor in preventing ischemic cardiovascular events with antimicrobial agents (109, 116, 151), probably due to its complex nature as a focal infection (109).
7. Animal studies show that long-term systemic challenge with *P. gingivalis* can accelerate atherogenic plaque progression (152).

Porphyromonas gingivalis

P. gingivalis is one of the major periodontal pathogens. From the infected oral epithelium, *P. gingivalis* uses several mechanisms (Figure 15) to initiate the atherosclerosis-related vascular inflammation (153) (Figure 9). *P. gingivalis* initiates macrophage secretion to inflammatory cytokines (154), and it is considered as an important candidate for cardiovascular diseases. The elevated antibody levels to the pathogen are shown to increase the risk for stroke (146, 147), and coronary disease (143-145). In addition, several components of *P. gingivalis*, e.g., outer memberane vesicles (155), and gingipains (156) are shown to inhibit the IFN-γ-mediated MHC class II expression. Studies in animal models show that *P. gingivalis* is one of the targets in experimental vaccination for periodontitis, suggesting a reduction in the rate and severity of bone loss (157).

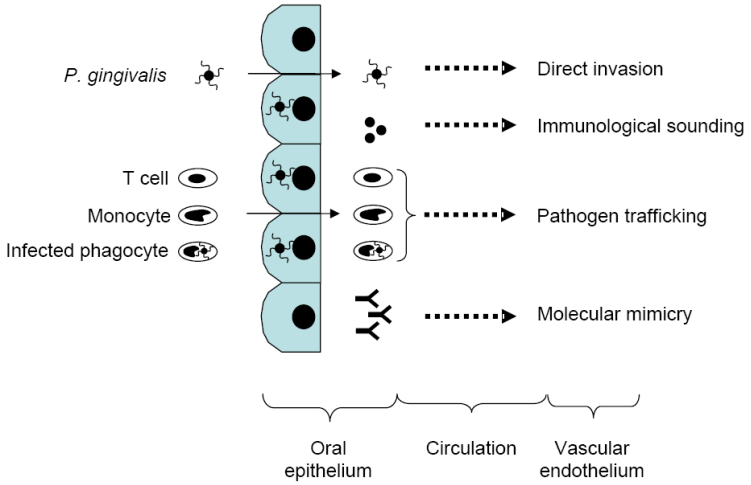


Figure 15. Mechanisms of *Porphyromonas gingivalis* linking oral epithelium and vascular inflammation. *P. gingivalis = Porphyromonas gingivalis*.

Briefly, the inflammatory and immune processes in periodontitis are complex. A variation in human susceptibility to periodontitis has long been accepted, but the pathological basis of this is poorly understood. Genetics and environmental factors play a role in the process (17, 18). Periodontitis (158) and *C. pneumonaie* infection (107) show both cell-mediated and humoral immune responses. Thus, genes that influence the infectious diseases (159) may affect the outcome of both *C. pneumoniae* infection, and periodontitis (17, 18). Furthermore, it has been speculated that periodontal disease could be a risk for cardiovascular disease in individuals, who have weaker immune responses against infections; this may be due to, e.g., low grade infections or genetic reasons (160).

5.4. Genes involved in coronary artery disease

CAD is a multifactorial disease; it shows an interaction with environmental and genetic factors (Figure 16).

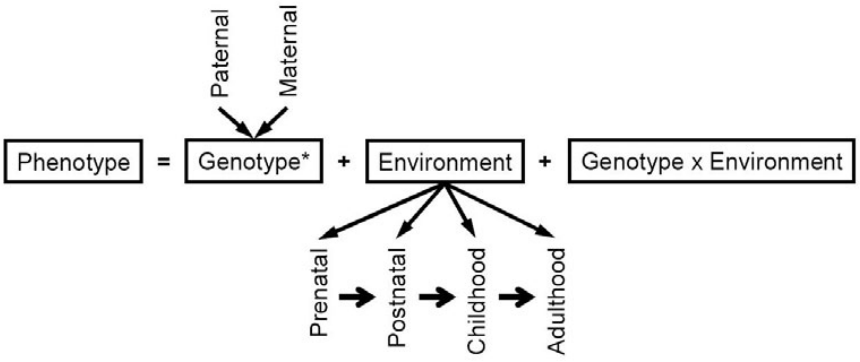


Figure 16. Classical relationship between phenotype, genotype and environment. For the CAD phenotype, risk factors that are influenced by environmental factors are explained in Section 5.1. Environmental effects are seen at all developmental phases of life. *Genotype which is inherited from parents includes gene-gene interactions. Genotype x Environment is the interaction between them.

Inheritability of CAD has been estimated to be greater than 50% (45). A two-fold risk for myocardial infarction was found for men with ≥1 affected parent or sibling, compared with men with no family history of coronary heart disease. Similarly, a 3.4-fold risk was found for males with ≥2 affected first degree family members. The corresponding risks for women were 2.1- and 4.4-fold, respectively (161). Twin studies (162, 163), and several other studies (19-21) provide evidence that genetics can partially explain CAD. This can be supported by the following 3 hypotheses (164):

1. The risk for CAD is influenced by the fetal development, as well as other factors acting on the period from fetal development to early adulthood.
2. The risk for disease evolves over life-time, and the influence of other specific factors is often conditioned by the presence of other (or later) exposures. For example, people with certain socioeconomical status are more prone to have modifiable risk factors, e.g., hypertension, diabetes, hypercholesterolemia, and obesity. These diseases are also genetically influenced.
3. Genetic and environmental factors interact to cause disease.

Genes that are shown as risks for CAD

Genes that are involved in different pathways leading to CAD are important. Polymorphisms or mutations in such genes have been shown to modify the risk for CAD (165). Examples of these pathways and genes (in parenthesis) are as follows:

1. Lipoprotein metabolism (e.g., apolipoprotein genes),
2. Homeostasis of blood pressure (e.g., angiotensin genes),
3. Glucose metabolism (e.g., insulin, and insulin receptor genes),
4. Hemostasis (e.g., coagulation factor genes),
5. Inflammatory response genes (e.g., cytokine genes),
6. Oxidation mediators (e.g., paraoxonase genes),
7. Adhesion mediators (e.g., cell adhesion molecule genes,),
8. Growth factor mediators (e.g., vascular endothelial growth factor genes),
9. Gene regulatory factors (e.g., interferon regulatory factor genes),
10. Others.

In the course of mapping chromosomal locations, exciting findings have been recently made in genetic studies of CAD. Some important gene(s) or gene regions have been shown as risk or susceptibility genes for CAD (Table 3).

Table 3. Recently identified susceptibility genes/loci for coronary artery disease in genome wide scan studies.

Chromosomal location	Gene	Trait	Ethnic group	Study reference
2q21.1-22	?	CAD	White (Finland)	(166)
Xq23-26	?	CAD	White (Finland)	(166)
16p13	?	CAD	Indo-Mauritians (India)	(167)
6p21	LTA	MI	Japanese	(168)
2q36-37	?	ACS	White (Australia)	(169)
14q	?	MI	White (Germany)	(170)
15q26	MEF2A	CAD, MI	?	(171, 172)
1p34-36	?	MI	White (USA)	(173)
3q13	?	CAD	White (USA)	(174)
13q12-13	ALOX5AP	MI, stroke	White (Iceland)	(175)
9p21.3	rs1333049	CAD	WTCCC and German	(176)
6p25.1	MTHFD1L	CAD	WTCCC and German	(176)
2p36.3	?	CAD	WTCCC and German	(176)
9p21	?	CHD	White (Caucasians)	(177)
9p21	?	MI	White (Caucasians)	(178)
8p	MSR1, FDFT1, FGL1, GATA4	CHD	French Canadian	(179)
1p31-32	?	CAD	?	(180)

See Appendix for abbreviations.

Ozaki K et al (168) carried out a genome-wide case-control association study using 92,788 gene-based single nucleotide polymorphisms with 94 Japanese patients with myocardial infarction. Positive single nucleotide polymorphisms with a nominal significance P value of 0.01 were then genotyped in 1,133 MI cases and 1,006 controls. Three single nucleotide polymorphisms in the LTA gene (see Section 5.4.1 for details) were found to be associated with a high risk of myocardial infarction (168). The LGALS2 (encoding gene is located in chromosome 21q13.1) regulates the extracellular secretion of LTA; and the intron-13279(C->T) variation of LGALS2 is signigicantly associated with a high risk of myocardial infarction (odds ratios vary from 1.2 to 1.6, based on comparisons made) (181; Table 4). Similarly, the MHC2TA (also known as CIITA, encoding gene is located in chromosome 16p13) regulates the expression of MHC molecules, and the MHC2TA-168(A->G) is also associated with a high risk of myocardial infarction (OR = 1.44, 95% CI = 1.15 to 1.8, P = 0.002) (182). Here, those genes which are not located in the MHC region, but are functionally related to the MHC genes (e.g., LGALS2, MHC2TA), are considered as “MHC regulating” genes/loci. In addition to these MHC regulating genes, several other studies (Table 4) also indicate the importance of the MHC region in CAD. Among these associated MHC genes, C4A*Q0 shows 1.9 and C4B*Q0 shows 1.5 odds ratios for patients with CAD undergoing coronary artery bypass when compared to controls (26).

Table 4. MHC genes and polymorphisms in MHC regulating loci/or associating genes linking to coronary artery disease.

Locus	Genes	Main findings	Association	Study reference
MHC	**TNF-α**	-308A	No	(183)
		-863A and -308A	No	(184)
MHC	**TNF-α and C4**	TNF-α -308A + C4A*Q0	Yes	(26)
		TNF-α -238A + C4A*6	Yes	(26)
MHC	**LTA**	252(a/g)	No	(184)
		252g	Yes	(185)
		10A, 252G and 804A	Yes	(168)
		252(A/G) or 804(C/A)	No	(186)
		804(C/A)	Yes	(187)
			Yes	(188)
MHC	**HLA-DR**	DR 3 and DR 13 with	Yes	(189)
		C. pneumoniae and LP(a)	No	(190)
		DR 2	Less prevalent	(191)
MHC	**HSP 70-1**	-110A	No	(192)
MHC	**HFE**	282Tyr or 63Asp	No	(193)
			No	(194)
		282Tyr	Yes	(195)
			Yes	(196)
			Yes	(197)
			No	(198)
			No	(199)
			Increased risk	(200)
MHC	**C4**	C4B*Q0	Yes	(10, 24)
		C4 null	No	(201)
			No	(202)
		C4A*Q0	Yes	(26)
MHC regulating[1]	**LGALS2**	3279(C/T)	Yes	(181)
	MHC2TA	-168(A/G)	Yes	(182)

See Appendix for abbreviations. [1] Those genes which are not located in the MHC region, but are functionally related to the genes in the MHC region (e.g., LGALS2, MHC2TA), are considered as MHC regulating genes/loci. LGALS2 coding gene is located in chromosome 22q13.1, while MHC2TA in chromosome 16p13.

In this thesis, the following MHC genes were studied: LTA, complement (C4A, and C4B), and HLA classical genes (HLA-A, -B, and -DRB1). Their genetic properties and potential roles in atherosclerosis or CAD are explained below or in the corresponding sections.

5.4.1. Lymphotoxin alpha

LTA is one of the genes in the TNF gene cluster located in the MHC class III region. The TNF gene cluster includes LTA, TNF and lymphotoxin beta cytokine-encoding genes. Ozaki K et al (168) performed a genome-wide case-control association study and showed three single nucleotide polymorphisms in LTA gene [exon 1 +10(G/A), intron 1 +252(A/G), exon 3 +804(C/A) coding Thr26Asn] associating with a high risk of myocardial infarction when these variations were homozygous. Odds ratios of the association for LTA-10(G/A), +252(A/G), and +804(C/A) were ~1.8, ~1.7, and ~1.8, respectively. In addition, the LTA+252(A/G) shows a 1.5-fold increase in the expression of LTA, and the Thr26Asn shows a 2-fold increase in the expression of adhesion molecules and cytokines, e.g., vascular cellular adhesion molecule-1, intercellular adhesion molecule 1, TNF, IL-1A and IL-1B, and selectin-E (168). This finding was further

supported by another study performed in a Caucasian population, showing a positive association of single nucleotide polymorphism Thr26Asn (187). Several other studies have also shown LTA as a susceptibility gene for coronary disease (185, 188, 203).

The LTA mediated immune responses in CAD have shown to be of importance also by Ozaki K et al (181). This study showed that a single nucleotide polymorphism located in the intron 1 of the LGALS2 gene [3279(C/T)] associated with myocardial infarction (odds ratios vary from 1.2 to 1.6, based on comparisons made) (Table 3). The LGALS2 gene encodes galectin-2, a member of the galactose-binding lectin family, which regulates the extracellular secretion of LTA. Repression of galectin-2 decreases or inhibits the LTA secretion, leading to less inflammation and reduced risk for cardiovascular diseases (181). The odds ratio for the association was between 1.2 and 1.6.

However, an independent, case-controled association study in a separate Japanese population (patients with myocardial infarction, n = 1,891; controls, n = 1,798) failed to identify any association of LTA+252(A/G) or Thr26Asn with myocardial infarction (186). This may indicate the need for further examination of the association.

Lymphotoxin alpha immunology

LTA is a member of the TNF family of cytokines, and is expressed and released mainly by CD4 Th1 cells, NK cells, and activated B cells (204). The secretion of LTA is regulated by different cytokines (e.g., IL-2, IL-4, and IL-7) (205, 206), and also by galectin-2 (181). LTAs are expressed as soluble homotrimers (207) and membrane bound heterotrimers composed of LTA and lymphotoxin beta (208). Their signalling specificities (208-210) are shown in Figure 17. The cellular signalling pathway followed by the members of the TNF superfamiliy leads to complex cellular responses (209), including the activation of nuclear factor κβ.

LTA and TNF are structurally related to each other and they share the overlapping receptor specificity and biological activities, and might have distinct roles in the immune system. LTA mediates a wide range of immune and inflammatory responses that can cause cell death or differentiation. Involvement of LTA in infectious diseases is complex (211).

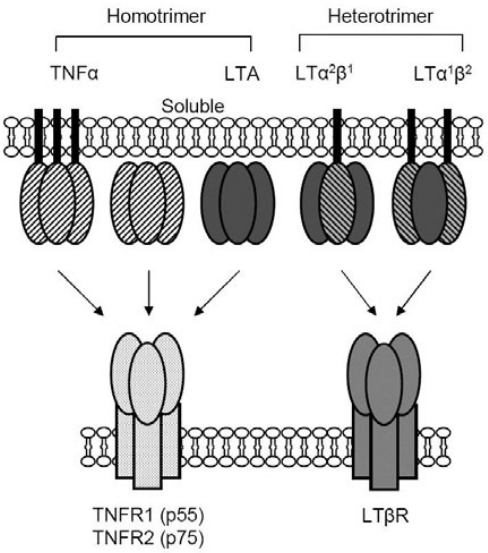


Figure 17. Tumor necrosis factor alpha and lymphotoxin alpha receptor specificity. See Appendix for abbreviations.

5.4.2. Complement system and C4 null alleles

The complement system functions with more than 30 different plasma and cell surface proteins. These proteins are activated in a cascade sequence; they generate several other molecules (Figure 18), and are involved in many aspects of the inflammatory response. The complement system can also turn its destructive capabilities against host cells and it is involved in numerous diseases and pathological conditions. It mediates chemotaxis effects, opsonisation of particles or micro-organisms, stimulates the release of enzymes from leukocytes, induces vasodilatation, enhances vascular permeability, and influences the cytokine network and the coagulation system (22, 23, 212).

The complement system has three different pathways (Figure 18). The antigen-antibody complexes initiate the classical pathway of activation, the alternative pathway by polysaccharides on the microbial surface, and mannan-binding lectin pathway which is activated by the mannose-containing proteins and carbohydrates on microbes. All three pathways merge at the activation of C3, and lead to the common pathway. The common pathway forms a membrane attack complex, which makes a pore on the cell surface, leading to death by osmotic lysis. The complement C4 plays a central role in the activation of the classical and lectin pathways, and in the elimination of circulating immune complexes (213).

The complement system displays varities of effects leading to local or systemic inflammation reactions (Figure 19), and many inflammatory, autoimmune, neurodegerative, and infectious diseases have been shown to be associated with excessive complement activity (212). These diseases include, e.g., infectious disease, sepsis, systemic lupus erythematosus, multiple sclerosis, transplant rejection, cancer, stroke, Alzheimer's disease, asthma, Crohn's disease, rheumatoid arthritis, and also myocardial infarction (212).

Studies indicate that the complement cascade is activated during vascular inflammation contributing to the development of atherosclerosis (214-216). Local activation of the complement system has been observed in myocardial infarction. Activated fragments, regulatory proteins, and complement receptors have been detected in atherosclerotic lesions, and the deposition of C5b-9 has been shown to correlate with the disease state (216). In addition, increased circulating levels of activated complement components in plasma have also been shown, e.g., C3a, C5a, and C4a (214). Normal arterial intima lacks signs of complement activation. Modified lipoproteins and necrotic cells can activate the alternative and the classical complement pathways (Figure 18), and signs of complement activation via these pathways have been seen in atherosclerotic lesions (216). Similarly, C-reactive protein can bind to bacterial surfaces and to the q subunit of complement component C1, and activate the classical pathway. C-reactive protein can also bind to complement factor H. Complement factor H is a regulator of the alternative complement pathway, and it has been suggested to play a role in complement inhibition in atherosclerotic lesions (217). Factor H His^{402} allele (SNP rs1061170) influences the ability of complement factor H to bind C-reactive protein (218), and it associates with myocardial infarction (219). Furthermore, the combined presence of the His^{402} homozygotes and C-reactive protein haplotype (haplotype of CCC for SNPs rs1130864, rs1205, and rs3093068) is associated with a risk of myocardial infarction (220).

Complement activation can also facilitate the activation of the coagulation cascade, e.g., the insertion of membrane attack complex in cell membranes is accompanied by the formation of membrane vesicles on the cell surface. These vesicles express binding sites for factor Va and support prothrombinase activity (214). C5a is able to upregulate intercellular adhesion molecule-1 in endothelial cells (214). In addition, complement and its activation products have the ability to provoke stimulation, aggregation, and degranulation of polymorphonuclear leukocytes (214).

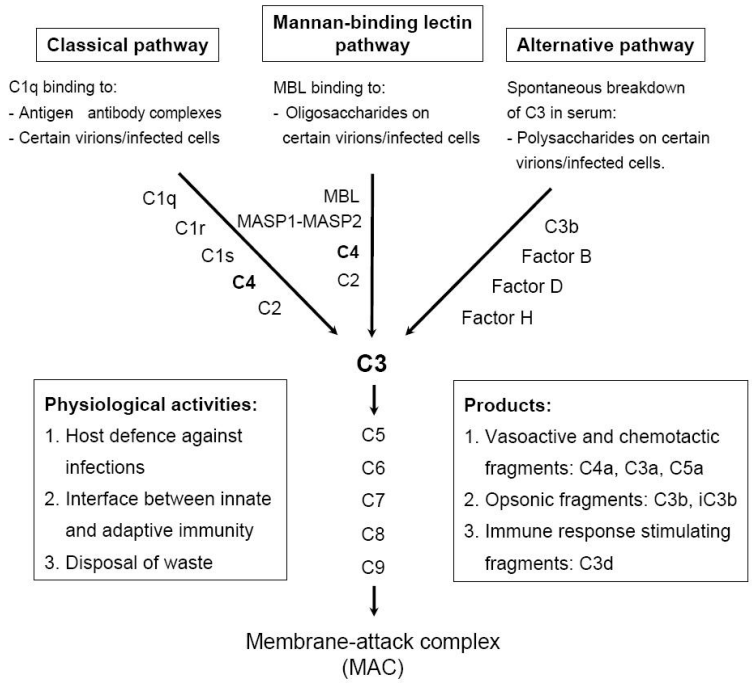


Figure 18. Model of complement cascade. See Appendix for abbreviations.

Complement factors C3 (encoded by C3 gene, located on chromosome 19p13) and C4 (encoded by C4 gene, located on chromosome 6p21) are mostly synthesized in liver but also in various other tissues (221, 222) such as in the infarcted heart (223). During the acute phase reaction, C3 and C4 protein levels in the blood are increased (224, 225), and they are consumed during complement activation, e.g., by damaged tissue, C-reactive protein complex, or immune complexes. Complement activation explains the increased consumption of C4. C4 may become consumed more by the classical pathway. Several other reasons can also result in the deficiency of complement components, e.g., increased consumption, insufficient or absent production. At the presence of C4 quantitative null alleles, the level of C4 synthesis may not match the need, partially explaining the C4 specific deficiency (30, 31). C4 producing organ-specific diseases, e.g., hepatitis, can also explain the insufficient or absent production of C4 proteins. Increased levels of serum C3 (27-29), and the presence of C4 deficiencies (C4A*Q0 and C4B*Q0) (10, 24-26) are shown as risks for CAD.

Therapeutic interventions aiming to modulate complement activity have also been adopted. These interventions include the C1 esterase inhibitor (inhibits the classical and lectin pathways), TP10 (also known as soluble complement receptor type 1, sCR1, which inhibits the C3 and C5 convertases), CRI (complement receptor type 1, inhibiting the alternative pathway), and pexelizumab (inhibiting C5 to prevent the activation of the terminal pathways) (227).

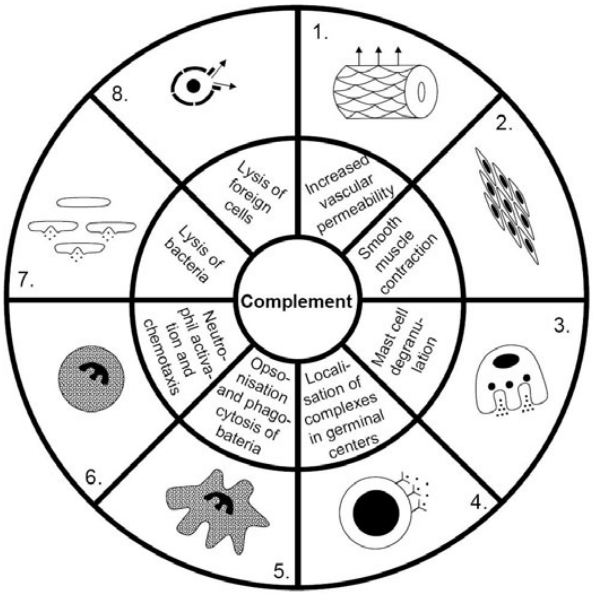


Figure 19. Roles of the complement system in the inflammatory reaction. Complement-derived peptides (C3a, C3b, and C5a) increase vascular permeability (1), cause smooth muscle contraction (2), activate leukocytes (6), and induce mast-cell degranulation (3). C3 assists both the localization of immune complexes in germinal centers (4) and opsonization and phagocytosis of bacteria (5). C5a is also a potent chemotactic factor for neutrophils and mononuclear phagocytes, which (6) phagocytose the opsonized micro-organisms. The membrane attack complex is responsible for the lysis of bacteria (7) and other cells recognized as foreign (8). Modified from: Walport M (226).

Complement C4 genetics, and null alleles

Complement C4 genes are located in the MHC class III region, and usually occur in two tandem duplications. One, coding for the serine proteases involved in the alternative pathway (C2 and factor B); and another, coding for the RP1 (also known as serine/threonine kinase 19, STK19), C4, cytochrome P450 family 21 (CYP21) and tenascin (TNX) genes forming a structure of RCCX (genetic module of RP-C4-CYP21-TNX) (30, 31). The RCCX is a complex genetic structure with segmental duplications forming several modules (Figure 20). Each contains a complement C4 gene. C4 genes are polymorphic and the C4 gene region is highly complex with the presence of different isotypes (C4A and C4B), size, and number of genes. C4 genes can be either long (20.6 kb) or short (14.2 kb) (228).

The C4A gene is responsible for the coding of the C4A protein (also known as Rodgers blood group), while C4B genes for the C4B protein (also known as Chido blood group). C4A has preference for amino groups and has immune complex clearance activity, whereas C4B has preference for hydroxyl groups and has more hemolytic activity than C4A (230).

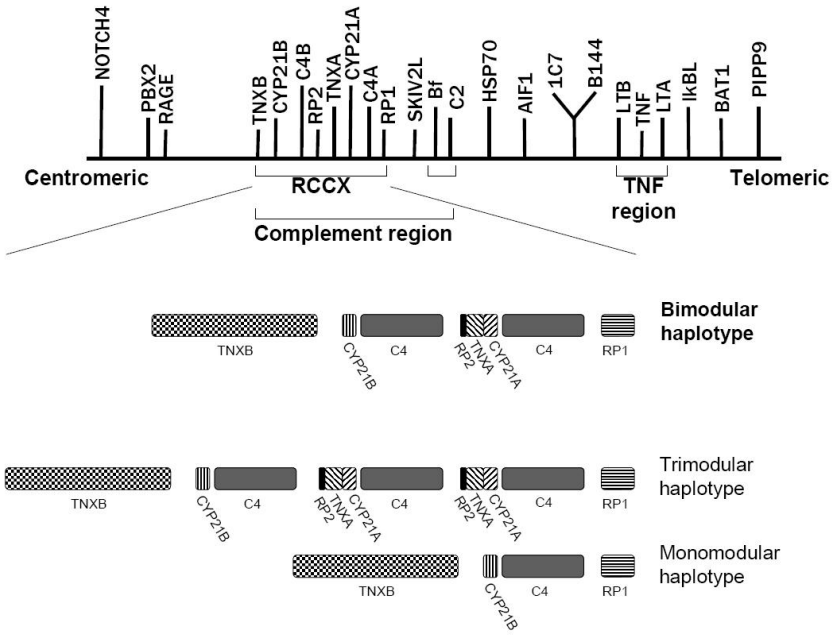


Figure 20. Location and copy number variations of the RP-C4-CYP21-TNX module. Bimodular haplotype is more frequent than monomodular and trimodular haplotypes. In an individual, different combinations can occur, e.g., bimodular-bimodular, bimodular-trimodular, bimodular-monomodular, monomodular-trimodular, etc. Among these variants, the most frequent variant is bimodular-bimodular (229). See Appendix for abbreviations.

Usually, gene deletion, point mutation, insertion or conversion events in the coding regions can cause early stop codons and encoding a premature protein with no functional activity (30, 31), known as C4 quantitative null alleles (presence of either of C4A*Q0 or C4B*Q0). The genetic background of C4 null alleles is complex. In HLA-B8− DR3 haplotype, C4A-CYP21A is often deleted (231). Homozygous deletion of long gene region (C4B-CYP21 and CYP21A-TNXA-RP2-C4B) has also been described in a Caucasian patient suffering from several pneumonias (232). Mostly in HLA-A2− Cw3− B60− DR6/DR2 haplptyoe carriers, a two-base pair (TC) insertion in exon 29 (codon 1213) has been described in mutant C4A genes (233, 234). Similarly, in HLA-A24− Cw7− B38− DR13 haplotype carriers, five exonic (9, 12, 13, 20, and 21) and five intronic (20, 28, and 29) sequence changes have been described in mutant C4A genes (235). In HLA-A30− B18− DR7 haplotype carriers, five exonic (9, 16, 20, 21, and 26) and 14 intronic (9, 19, 20, 21, 28, 30, 31) sequence changes have been described in mutant C4B genes (235). In HLA-B35− DR1 haplotype, gene conversion most often causes the transition of the C4B gene to C4A (231, 236). The rare complete C4 null haplotypes C4AQ0− C4BQ0 have also been found with a different HLA haplotype, but most frequently with the HLA-B60− DR6 haplotype (237).

Null alleles are common (238, 239), and associate with several diseases, e.g., systemic lupus erythematosus, multiple sclerosis, vitiligo, idiopathic membranous nephropathy, Henoch-Schönlein nephritis, Ehlers-Danlos syndrome, and coronary diseases (9, 10, 240); also, they can act as markers of gene rearrangements in class III unfavorable for pregnancy outcome (241).

Briefly, certain variations in the LTA gene (168) (see Section 5.4.1 for details), and C4 null alleles (9, 10) have been shown as risk factors for CAD. These genes may be inherited in linkage disequilibrium with HLA classical genes forming extended MHC haplotypes. However, no such study in relation to CAD is available. The C4A*Q0 and C4B*Q0 alleles usually accompany certain extended haplotypes, e.g., HLA-A1− B8− DR3 and HLA-A3− B35− DR1, respectively (231, 236, 242, 243). Such haplotypes may be better markers for CAD than a single gene of C4 or LTA.

5.4.3. Major histocompatibility complex region

There are various types of highly densed genes present in the MHC region. Functionally most of these genes are related to infection, inflammation, and immune responses.

Genetic organization

This region is located on the short arm of the human chromosome 6 (6p21). The first sequence based map of the MHC region described 224 gene loci with 3.6 Mb long gene sequences (244). A newly-defined extended MHC region covers a total of 7.6 Mb (245) (Figure 21). About 5% of the human genome can be attributed to segmental duplications, resulting in gene clusters (246), such as the MHC region (Table 5).

The compilation of information on gene number, structure and sequences, polymorphism, haplotype composition, and linkage disequilibrium complicate the MHC region (247). However, the MHC genes are categorized according to their functional involvement (Table 6), and carry a variety of functions in the regulation of both innate and adaptive immunity, with striking influence on the course of infection and inflammation (3).

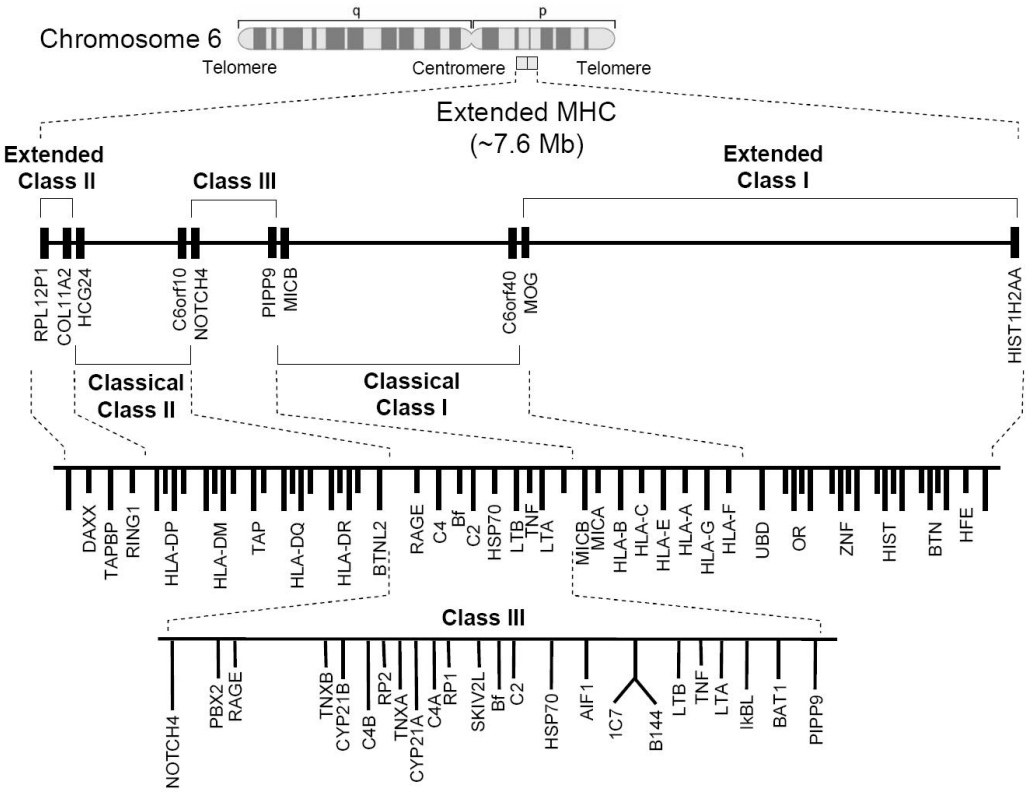


Figure 21. A schematic diagram of genes in the major histocompatibility complex region. The extended MHC region extends from histone cluster 1 H2aa (HIST1H2AA) to ribosomal protein L12 pseudogene 1 (RPL12P1). About 7.6 Mb with five subregions is shown: extended class I (HIST1H2AA to MOG, 3.9 Mb), classical class I (C6orf40 to MICB, 1.9 Mb), class III (PPIP9 to NOTCH4, 0.7 Mb), classical class II (C6orf10 to HCG24, 0.9 Mb) and extended class II (COL11A2 to RPL12P1, 0.2 Mb). See Appendix for abbreviations. Based on: Lokki ML (248), and Horton R et al (3).

Table 5. Gene clusters within the extended major histocompatiblity complex region.

Cluster type	Number of protein-coding loci	Number of psuedo-gene/transcript loci	Total number of loci
Gene superclusters			
Histone	55	11	66
HLA class I	9	17	26
tRNA	151	6	157
Butyrophilin	8	0	8
Olfactory receptor	14	20	34
Zinc finger protein	26	10	36
Gene Clusters			
Solute carrier 17A	4	0	4
Vomeronasal receptor	0	5	5
Tumour necrosis factor	3	0	3
Lymphocyte antigen-g	5	0	5
Heat shock protein	3	0	3
HLA class II	15	9	24
Total	**293**	**78**	**371**

The HLA class I gene supercluster is comprised of the classical (HLA-A, -B and -C), and non-classical class I genes (HLA-E, -F, -G, hemochromatosis and 12 pseudogenes) and class I-like genes (MHC class I polypeptide-related sequence A (MICA), and B (MICB), and 5 pseudogenes). Similarly, the HLA class II gene cluster comprises the classical (HLA-DP, -DQ, -DR and pseudogenes) and non-classical class II genes (HLA-DM and -DO). The class III genes contain several immunological genes, e.g., complement components (C4, C2, Bf), 21-hydroxylase (deficiency can lead to congenital adrenal hyperplasia), stress response (heat shock proteins, MICA, MICB), cytokines (TNF-α, LTA and LTB). See Appendix for abbreviations. Modified from: Horton R et al (3).

Table 6. Categories of immune response genes in the extended major histocompatibility complex region.

Category	Genes
Antigen processing/ presentation	HLA-A, -B, -C, -DMA, -DMB, -DOA, -DOB, -DPA1, -DPB1, -DQA1, -DQA2, -DQB1, -DQB2, -DRA, -DRB1, -DRB3, -DRB4, -DRB5; PRSS16; PSMB8, PSMB9; TAP1, TAP2, TAPBP; UBD
Immunoglobulin superfamily	AGER; BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A1, BTN3A2, BTN3A3, BTNL2; C6orf25; MOG
Inflammation	ABCF1; AIF1; DAXX; IER3; LST1; LTA, LTB; NCR3; TNF
Leukocyte maturation	DDAH2; LY6G5B, LY6G5C, LY6G6D, LY6G6E, LY6G6C
Complement cascade	BF; C2, C4A, C4B
Non-classical MHC class I	HLA-E, HLA-F, HLA-G; HFE
Immune regulation	NFKBIL1, RXRB, FKBPL
Stress response	HSPA1A, HSPA1B, HSPA1L; MICA, MICB

See Appendix for abbreviations. Modified from: Horton R et al (3).

Nomenclature of HLA genes

The HLA gene nomenclature is evaluated by the World Health Organization Nomenclature Committee. Certain genes are denoted by a letter (or letters) for locus (e.g., HLA-A, -B, -DR) and alleles are representated with the additional number. For example, HLA-A2 and HLA-A3 are alleles of the HLA-A locus; similarly, HLA-B7 and HLA-B35 are alleles of the HLA-B locus. Initially, alleles were defined by serological methods, which had its limitations. Advanced DNA based methods have identified sequence variations between alleles. In the current terminology, a locus is followed by a 2 digit number (e.g., HLA-A*01 and HLA-DRB1*01), or a 4 digit number (e.g., HLA-A*0101 and HLA-DRB1*0101), where the first two digits denote alleles and the next two subtypes of the allele.

5.4.3.1. Human leukocyte antigens

MHC molecules are expressed cell surface glycoproteins. The class I molecule consists of a heavy chain (α chain), which is non-covalently assembled with a light chain (β2-microglobulin, encoded by a gene located in chromosome 15q21). The polymorphic classical class I genes (HLA-A, -B, and -C) encode the α_1, α_2, and α_3 domains of the class I molecule (Figure 22). The MHC class I molecules are expressed by all nucleated cells. The class II molecules are composed of an α chain, which is non-covalently assembled with a βchain. The classical class II genes (HLA-DR, -DP, and -DQ) encode the α and β chain (Figure 22); among the class II genes, HLA-DRB1 is the most polymorphic. The classical class II genes are expressed by antigen-presenting cells (APC, e.g., dendritic cells, B cells, and macrophages) (249, 250). Exceptional nucleotide diversities in HLA-A, -B, -C, and -DRB genes influence in the variations of the epitope-binding sites in the peptide-binding groove in both class I and II molecules.

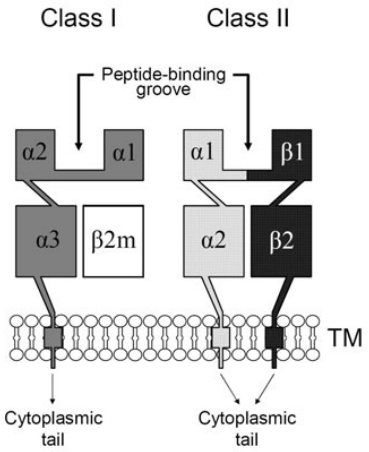


Figure 22. Major histocompatiblity complex molecules. Both molecules are structurally homologous but functionally different cell surface glycoproteins. Both molecules have three parts: extracellular (α and β domains), transmembrane, and a short cytoplasmic tail. The α1 and α2 in class I and α1 and β1 in class II molecules form the polymorphic peptide-binding grooves. β2m = β-microglobulin.

MHC molecules facilitate antigen recognition by T cells, thereby initiating the adaptive immune responses. For this, APCs process antigenic peptides which are bound to the peptide-binding groove (Figure 22), and presented to T cells. In the case of MHC class I, the peptides are most often bound by the side chains of their 2nd (P2), and 9th (P9) amino acids to the peptide binding groove (Figures 22, 23). Similary, the 1st (P1), 4th (P4), 6th (P6), and 9th (P9) are the binding amino acids in MHC class II molecule. Thus, the binding is higly specific (249, 250).

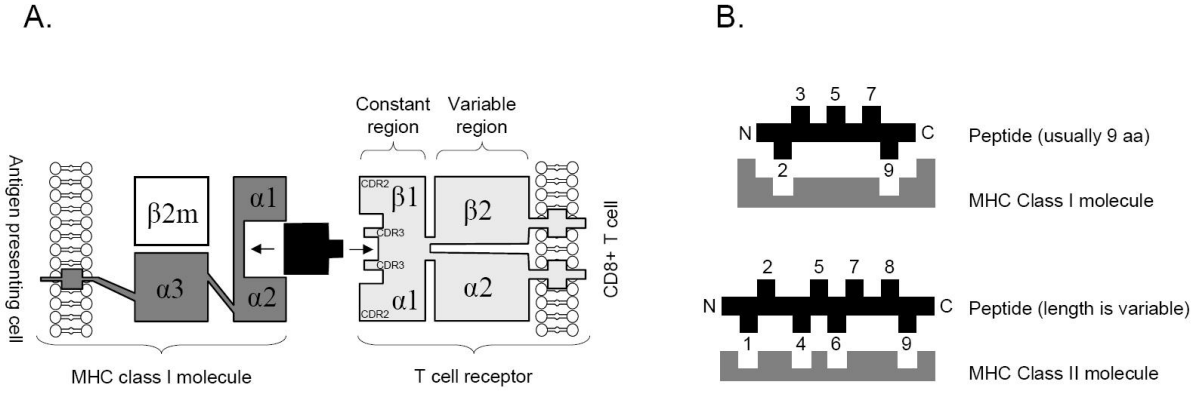


Figure 23. Major histocompatibility complex class I molecule - peptide - T cell receptor complex (A), and independent contribution of each amino acid of peptides in binding (B). In the variable region of the TCR molecule, three segments formed by the T cell receptor complementary-determining region 1 (CDR1), 2 (CDR2) and 3 (CDR3) show the highest degree of amino acid variability. See Appendix for abbreviations.

The NK cell receptor is involved in class I, and TCR in both class I and II interactions. The presence of CD4 or CD8 molecules on the surface of mature T cells is identified by T cell maturation in thymus (Figure 24). During this, the maturing thymocytes rearrange the TCR genes and posess both CD4 and CD8 molecules, but interactions with HLA molecules cause downregulation of one and upregulation of the other. This event

results in mature T cells with CD4+/CD8- or CD4-/CD8+ type (251). Generally, the CD4+ T cells become helper T cells, while the CD8+ T cells become cytotoxic T cells.

The CD4 or CD8 molecules interact as coreceptors with the MHC-peptide complex and help in the adhesion with TCR, and provide specific activating signals to T cells. No TCRs bind the MHC molecule alone or when complexed with an unrelated peptide; thus, this binding is a highly specific event. TCR consists of two polypeptide chains (α and β) with variable and constant regions (Figure 23).

Briefly, the complex of MHC molecule-peptide-TCR molecule is extremely specific, and variations of epitope binding sites exist for both MHC and TCR molecules.

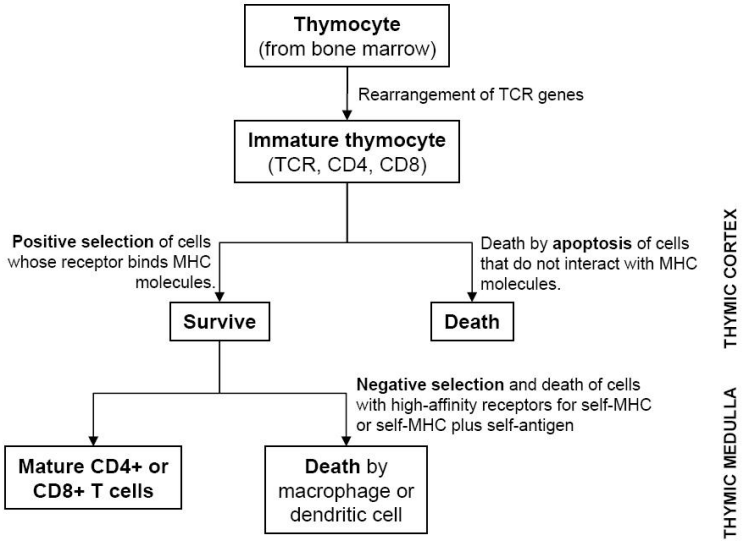


Figure 24. T cell maturation in thymus. Cells that survive both positive and negative selection leave the thymus and enter the periphery.

5.4.3.2. Antigen presentation and induced immune responses

The immune system

The immune system identifies foreign molecules as "nonself" and thereby destroys them. These nonself molecules are called antigens. They are usually protein fragments or carbohydrates. The immune response is an integrated host response to an antigen. The human immune system can usually be divided into two major divisions: innate and adaptive (or acquired) immune response. Pathogens that successfully enter the host must encounter the innate immune system first. This system is non-specific, and does not confer long-lasting immune response against a pathogen, but acts within hours, and also sends cytokine signals to the adaptive immune system. Usually, complement system, granulocytes, natural killer cells, macrophages, and phagocytosis belong to the innate immune system. On the other hand, the adaptive immune system is specific for a pathogen, allows a stronger immune response, and provides also the immunological memory.

For adaptive immune sytem, receptors need to go through gene rearrangement (e.g., B cell receptor, T cell receptor), and they recognize a wide variety of molecular structures (e.g., proteins, peptides). The adaptive immune system includes humoral and cell-mediated (or cellular) immune responses. Antibody-mediated immunity (e.g., B cells, antibodies, and memory cells) belongs to the humoral immune response, while cell-mediated immunity (e.g., T cells, cytokines, and B cell activation) mediates cellular immune responses.

Antigen presentation

Antigen binding by MHC molecules is a highly selective event. The MHC-peptide complex recognition by a T cell is an initial event for the immune response. For this event, antigens should undergo certain cellular processes in the APCs, and later they are transported to the cell surface for recognition by T cells. These cellular processes are divided into two distinct pathways: the endogenous (also known as class I pathway) and the exogenous (also known as class II pathway) pathways. The endogeneous pathway collects the fragments of proteins derived from intracellular pathogens, and then displays these antigens at the cell surface via the MHC class I molecule to CD8 T cells. Generally, class I molecules serve as security against the intracellular pathogens. On the other hand, the exogenous pathway collects the self or foreign components or pathogens that are endocytosed by APCs (e.g., dendritic cells, and macrophages) and displays these antigens at the cell surface via MHC class II molecules to CD4 T cells. Generally, class II molecules serve as security against the extracellular pathogens (249, 250).

The professional APCs (e.g., dendritic cells) can possess both class I and II pathways. In some situations (e.g., in autophagy), cells can transfer intracellular antigens into the class II pathway in addition to class I. Following the receptor-mediated endocytosis, B cells can process antigens and follow the class II pathway (252, 253). The CD4 T cell recognizes the peptide, becomes stimulated and releases lymphokines. These lymphokines stimulate the antigen-presenting B cells to enter the cell cycle, and grow into a clone of plasma cells. Plasma cells then synthesize immunoglobulins that are secreted into the cell surroundings. Lipid antigens are presented to T cells by a cell-surface molecule designated CD1, which is a non-MHC lineage of the antigen-presenting molecule (254). CD1 molecules form an antigen-binding groove similar to the MHC molecules. It has been demonstrated that CD1-restricted T cells have a pathogenic role in atherosclerosis (255).

In order to become activated, T cells must not only bind to the MHC-peptide complex with its TCR (signal one) but also receive a second signal (also known as “costimulation”) from the APC cell. For the second signal, one of the most important costimulators is the B7 molecule on the surface of the APC cell. The ligand of B7 molecule is CD28 molecule on the surface of a T cell. B7-CD28 binding provides the second signal needed to activate the T cell (256, 257). Under normal physiological conditions, T cells do not respond unless they receive the second signal. In the absence of signal two, signal one results in unresponsive T cells or even self-destruction by apoptosis.

Immune responses and atherosclerosis

During antigen presentation, the MHC class I molecule shows restrictions to CD8, and class II to CD4 T cells (249, 250). Activation of CD8 T cells initiates cytolysis and/or secretion of cytokines (258, 259), while activation of CD4 T cells initiates the secretion of a wide range of cytokines (260) (Figure 25). Their involvement in atherosclerosis is explained in Section 5.2.

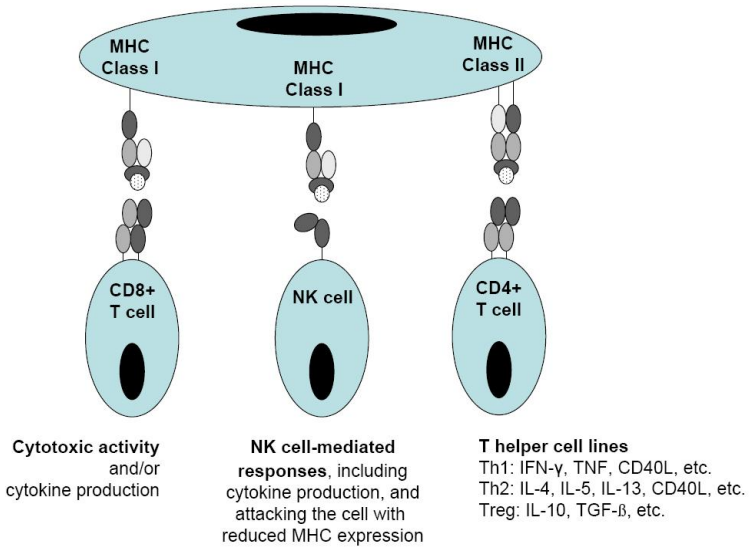


Figure 25. Immune responses by major histocompatibility complex molecules.
See Appendix for abbreviations.

Natural killer cells also bear receptors in addition to many other pattern recognition receptors which recognize MHC class I molecules and act as inhibitory molecules (killer inhibitory receptor) (Figure 25). This prevents the killer activity of NK cells, preventing the MHC class I-bearing cells to be lysed. When MHC expression is downregulated or unrecognized (see Section 5.4.3.3 for details), they become the target for natual killer cell-mediated killing (261, 262).

In the atherosclerotic lesion, CD4 T cells dominate over CD8 T cells, and the ratio of CD4/CD8 T cells is similar as in the peripheral blood (49). However, CD4 T cell responses are considered as proatherogenic (66) (see Section 5.2 for details), but evidence supporting the significant role of CD8 T cells in atherosclerosis is also increasing. CD8 T cells are present in the lesions, and can exert different effects in atherosclerosis, including cytolysis and secretion of proinflammatory cytokines (258, 259). IFN-γ elicits proatherogenic effects (66), its main source is Th1 cells, but IFN-γ can also be produced by CD8 T cells, NKT cells, NK cells, and IL-18 stimulated smooth muscle cells (258, 259). Furthermore, in a transgenic mouse model, the activation of CD8 T cells has lead to severely accelerated atherosclerosis (263).

Cross-reactivity between self and nonself molecules leads to antiself immune responses (264), known as molecular mimicry. Both the microbial heat shock 60/65 kDa protein and human 60 kDa protein are expressed in human atherosclerotic lesions (50) and they can activate the host immune responses directing towards molecular mimicry (Figures 8, 9). Increasing evidence supports that not only CD4 T cells, but also CD8 T cells play important roles in the pathogenesis of several autoimmune disorders, e.g., rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, etc (265).

5.4.3.3. Regulation of major histocompatibility complex molecule expressions

During an inflammatory response or infections, impaired MHC expressions are detected. However, it is not well understood which cell types are responsible for the impaired expression of MHC molecules in atherosclerosis. In general, various mediators as well as genetic componets regulate the expression of MHC molecules.

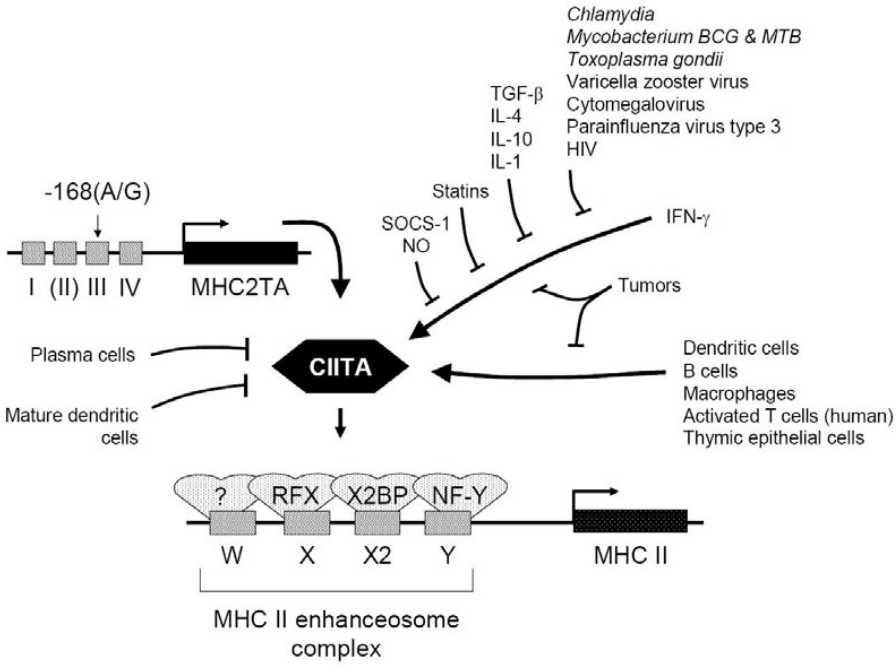


Figure 26. Activation or silencing of class II major histocompatibility complex transactivator gene. Activating or inhibiting influences on CIITA expression are indicated. See Appendix for abbreviations.

The mediators which regulate the expression of MHC molecules are shown in Figure 26. Several cytokines (e.g., IFN-γ, interleukins, and TGF-β) can modulate the class II expression (266, 267). For example, IFN-γ increases the expression of class I molecule, while thyroid-stimulating hormone decreases it (268). As a genetic component, the expression of class II molecules is regulated by four promoter elements: W (or S), X, X2, and Y boxes (Figure 26). The regulatory factor X binds the X box, and the X2-binding protein and nuclear factor Y proteins bind X2 and Y boxes (269). Several proteins can bind the W box *in vitro*, including

regulatory factor X (270), but the functional relevance of W box-binding protein is not known. The binding at the enhanceosome is managed by the class II MHC transactivator (CIITA encoded by the MHC2TA gene).

The CIITA is regulated in response to different components (266, 271). Four promoters (I, II, III, and IV) are believed to control the MHC2TA gene expression (Figure 26). Promoter II shows low activity and its role is not known. Cell-type specific modulation of MHC2TA expression is controlled by promotors I, III, and IV (269). In a case control study (387 patients with myocardial infarction as cases and 387 controls), the -168(A/G) variation in promoter III was associated with increased susceptibility to myocardial infarction (OR = 1.44, 95% CI = 1.15 to 1.18, P = 0.002) (182).

5.4.3.4. Disease associations with major histocompatibility complex genes

In addition of controling immune responses in transplant acceptance, the MHC genes represent a primary target for disease gene discovery efforts. Various MHC genes have multiple functions and they are the most polymorphic region in the human genome, which leads them to associate with human diseases. Most of the MHC gene-associated diseases are chronic inflammatory, autoimmune or infectious in nature (Table 7). In certain cases the association is very strong, and implies the obvious immune pathology (272).

Although the compilation of information on gene number, structure and sequences, polymorphism, haplotype composition, and linkage disequilibrium complicate the understanding of a dynamic view of the MHC region (247), significant studies have been performed to understand it. The majority of genes in the MHC region take part in adaptive and innate immunity, with essential function in inflammatory reactions and in protection against infections (3, 245, 249, 250, 273).

Extreme levels of polymorphism in the MHC genes reflect not only the epitope binding sites but also the promoter region that affects the expression of the MHC molecule. On the other hand, highly polymorphic MHC genes form conserved extended haplotypes with strong linkage disequilibrium (4), and information of such haplotypes provides new and stronger tools for gene mapping (7, 8). Examples of such haplotypes are HLA-A1− B8− DR3, and HLA-A3− B35− DR1, also known as ancestral haplotypes 8.1 and 35, respectively. The HLA-A3− B35− DR1 haplotype has the highest frequency in the Finnish population (274-276), and the C4B*Q0 seems to accompany this haplotype (242); however, its importance is not known. Similarly, the HLA-A1− B8− DR3 is common in Northern Europe and the C4A*Q0 seems to accompany this haplotype. The HLA-A1− B8− DR3 haplotype is associated with several immunological disorders, e.g., type I diabetes, myasthenia gravis, celiac disease, and rapid disease progression of HIV infection (277).

Table 7. Association of various human leukocyte antigen markers with autoimmune diseases and infections.

Associated diseases	HLA molecules or markers	HLA alleles[1]	Effects
Ankylosing spondylitis	B27		Susceptibility
Behcet's syndrome	B51		Susceptibility
Birdshot retinochoriodopathy	A29		Susceptibility
Coeliac disease	DR3, DR7, DR11		Susceptibility
	DQ2	HLA-DQA1*0501/DQB1*0201	Susceptibility
	DQ8	HLA-DQA1*0301/DQB1*0302	Susceptibility
Dermatitits herpetiformis	DR3		Susceptibility
Goodpasture's syndrome	DR2		Susceptibility
Graves' disease	DR3		Susceptibility
Hashimoto's thyroiditis	DR11		Susceptibility
Idiopathic Addison's disease	DR3		Susceptibility
Insulin-dependent (type 1) diabetes mellitus	DR2		Protective
	DR3, DR4		Susceptibility
	DR4.1	HLA-DRA1*0101/DRB1*0401	Susceptibility
	DR4.3	HLA-DRA1*0101/DRB1*0403	Protective
	DR.4.5	HLA-DRA1*0101/DRB1*0405	Susceptibility
	DQ2	HLA-DQA1*0501/DQB1*0201	Susceptibility
	DQ6	HLA-DQA1*0102/DQB1*0602	Protective
	DQ8	HLA-DQA1*0301/DQB1*0302	Susceptibility
Idiopathic membranosus glomerulonephritis	DR3		Susceptibility
Multiple sclerosis	DR2a	HLA-DRA5*0101/DRB5*0101	Susceptibility
	DR2b	HLA-DRA1*0101/DRB1*1501	Susceptibility
	DQ6.2	HLA-DQA1*0102/DQB1*0602	Susceptibility
Myasthenia gravis	B8, DR3		Susceptibility
Narcolepsy	DQ6.1	HLA-DQA1*0102/DQB1*06011	Protective
	DQ6.2	HLA-DQA1*0102/DQB1*0602	Susceptibility
Pemphigus vulgaris (among Ashkenazi Jews)	DR4		Susceptibility
Postpartum thyroiditis	DR4		Susceptibility
Psoriasis vulgaris	Cw6		Susceptibility
Reactive arthropathy, including Reiter's syndrome	B27		Susceptibility
Rheumatoid arthritis	DR1	HLA-DRA1*0101/DRB1*0101	Susceptibility
	DR4.1	HLA-DRA1*0101/DRB1*0401	Susceptibility
	DR4.2	HLA-DRA1*0101/DRB1*0402	Protective or neutral
Sicca syndrome	DR3		Susceptibility
Systemic lupus erythematousus	DR3		Susceptibility
Infectious diseases			
AIDS	B35		Susceptibility
	B57		Protective
Aspergillus	B35		Susceptibility
Epstein-Barr	B35		Susceptibility
Hepatitis B	B35, DR7		Susceptibility
	DRB1*1302		Protective
Hepatitis C	B35		Susceptibility
	DRB1*1101		Protective
Herpes simplex	B35		Susceptibility
Influenza A	B35		Susceptibility
Leprosy	DR2		Susceptibility
Malarial anaemia	DRB1*1352		Protective
Malaria, severe	B53		Protective
Mycobacterium tuberculosis	B35		Susceptibility
Pulmonary tuberculosis	B8, DR2		Susceptibility
Typhoid fever	DRB1*04		Protective

[1] HLA alleles indicate haplotypes that code the HLA molecules. HLA-B35 is focused for the infectious disease. AIDS = Aquired immune deficient syndrome. Modified from: Klein J et al (250), Cooke GS et al (273), and Jones EY et al (278).

The HLA haplotype patterns in different populations differ substantially affecting on the outcome of human diseases. Individuals with certain HLA haplotypes may be protected from certain infectious diseases, while individuals with non-resistant haplotypes will get infected (279). During the early stage of atherosclerosis,

varieties of antigens are presented by APCs to T cells, and oxLDL being one of them. APCs internalize the oxLDL. After proteolytic processing, fragments of the protein component of LDL, apolipoprotein B, binds nascent MHC class II molecules and are transported to the cell surface for T cell recognition. Apolipoprotein B fragments are among the peptides most frequently displayed by HLA-DR molecules in cultured human lymphoblastoid cells (66, 280, 281).

Briefly, it is not surprising that genes from the MHC region influence the susceptibility to diseases having strong immunological background, as listed in Table 7. CAD is an inflammatory disease (66). Studies show that the MHC genes (e.g., LTA in Table 3, C4B*Q0 in Table 4) or MHC-related genes (e.g., MHC2TA, LGALS2, and, C4A*Q0 in Table 4) associate with CAD. However, we do not know which are the gene(s) and by which mechanism they influence the cause of the disease. The associated genes might link to each other or with HLA classical genes forming an extended MHC haplotype. Such a haplotype may carry multiple biological effects, based on genes involved.

5.5. Statins as immune modulators

Statins (3-Hydroxy-3-Methyl-Glutaryl-CoA reductase inhibitors, effective lipid-lowering agents) exhibit strong immune modulatory effects. Statins are proposed to act in the prevention of atherosclerosis with four different mechanisms (282): by improving endothelial function, modulating inflammatory responses, maintaining plaque stability, and preventing thrombus formation.

Statins seem to exhibit the immunemodulatory effects in the balance of cytokines. In patients with unstable angina who received statins, the Th1/Th2 ratio (estimated by the ratio of CD4+ [IFN-γ^+] [IL-4^-] cells / CD4+ [IFN-γ^-] [IL-4^+] cells) significantly decreased when compared with those who did not receive statins, suggesting decreased Th1 responses in those receiving statins. Statins can increase the secretion of Th2-related cytokines, and decrease that of Th1-related cytokines (283).

On the other hand, statins can inhibit the expression of MHC class II molecules. Statins suppress the IFN-γ-induced MHC class II expression as well as the MHC class II mediated T-cell activation. This is due to the inhibition of promoter IV of CIITA (Figure 26). Atorvastatin (284) and simvastatin (285) are shown to suppress the IFN-γ-induced MHC class II expression. Atorvastatin treatment leads to downregulatioin of HLA-DR molecules (286). In addition, statins can reduce the cell surface immunoregulatory molecules, e.g., MHC class I molecule, CD3, CD4, CD8, CD28, and CD40 (287). However, in myopathy, those who received statins showed upregulation of MHC class I expression (283).

Seeking primary and secondary prevention for atherosclerosis, several statin trials have been perfomed, e.g., EXCEL (288), 4S (289), AFCAPS/TexCAPS (290), ASCOT (291), ASTEROID (292), SPARCL (293), etc.

These trials have all showed favorable human outcomes (lowering lipid levels), and only ASTERIOD showed evidence of atherosclerotic regression (slight).

Briefly, apart from their lipid lowering effects, statins seem to have both anti-inflammatory and anti-microbial properties. However, the use of statins needs to be long enough (probably several years) to have any effects on the prevalence of chronic diseases, e.g., periodontitis, CAD, probably also on chronic *C. pneumoniae* infection.

6. AIMS OF THE STUDY

The aims of the present study were to evaluate:

1. whether the MHC region contains genes that associate with CAD;
2. whether the MHC genes associate with *C. pneumoniae* infection in patients with CAD;
3. whether the MHC genes associate with periodontitis and major periodontal pathogens in patients with CAD;
4. whether serum C3 and C4 protein concentrations predict the recurrent cardiovascular end-points in patients with CAD.

7. STUDY SUBJECTS

Three separate Finnish groups formed two independent studies: HTx recipients, patients with ACS, and controls (consecutive age- and sex-matched healthy blood donors to patients with ACS) (Figure 27). Study I includes all study subjects, study II includes patients with ACS and controls, studies III and IV include patients with ACS.

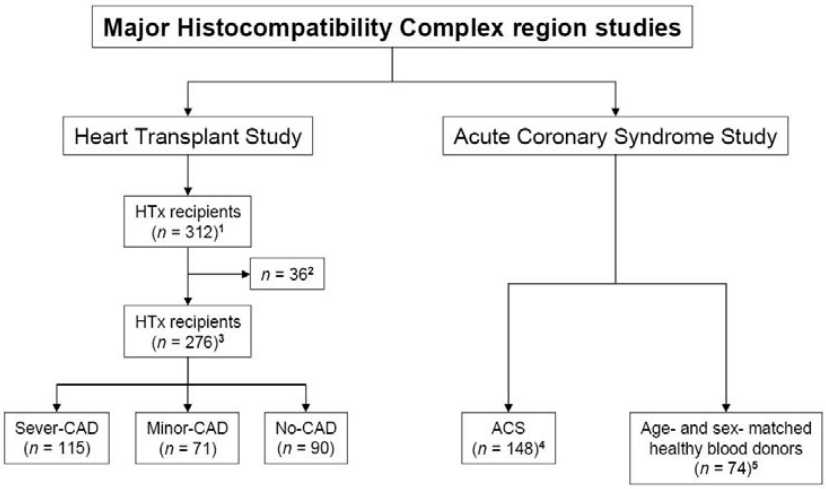


Figure 27. Studies and study subjects. ACS = Acute coronary syndrome, CAD = Coronary artery disease, HTx = Heart transplant.
[1] HTx recipients comprised all patients who received a heart transplant from the beginning of HTx in Finland in February 1985 to March 2003.
[2] Because of insufficient data, 36 were excluded.
[3] Indications to receive a heart transplant were: inoperable CAD leading to heart failure (n = 100), or severe forms of non-ischemic cardiomyopathy, myocarditis, congenital and valvular heart defects, and malignant arrhythmias (n = 176). Based on the pathological specimens of the removed heart, the HTx recipients were divided into three groups following the atherosclerotic lesions found: Severe-CAD (≥50%), Minor-CAD (<50%), and No-CAD (0%).
[4] Patients with ACS (n = 148) were recruited from September 1998 to December 2002 (114). Among them, study I includes 100, study II includes 100, study III includes 106, and study IV includes 148 patients. For study III, dentate patients with ACS (n = 106) were divided into two groups as a group of "non-periodontitis", comprised of no radiographic periodontal attachment loss, and as a group of "periodontitis", comprised of radiographic periodontal attachment loss.
[5] Consecutive age- and sex-matched healthy blood donors were collected from the Finnish Red Cross Blood Transfusion Service, Helsinki, and they severed as controls for the ACS study. Any of the followings clinical characteristics (previous or ongoing) were not accepted for blood donation: hypertension, coronary heart disease, myocardial infarction, arrhythmias, rheumatic fever or any other cardiovascular disease, or bypass or valvular surgery (http://www.veripalvelu.redcross.fi/). The criterion, which was used to select the blood donors as control subjects for this study, was the age- and sex-matching to patients with ACS.

Patients with ACS (n = 148) were collected during September 1998 to December 2000 from nine different central hospitals in Finland for a placebo-controlled clarithromycin study on ACS (114). Patients with prolonged chest pain with changes in ST-wave indicating unstable angina (n = 43) or non-Q-wave acute myocardial infarction (n = 105) were enrolled. ST-elevation myocardial infarction was an exclusion criterion. For inclusion, patients had to have clear symptoms of angina with electrocardiographic evidence of myocardial infarction. Patients who met the anginal pain inclusion criteria but none of the

electrocardiographic criteria were eligible to enter the trial if their cardiac enzymes were consistent with the occurrence of myocardial infarction. Patient treatment, follow-up, and end-points were as described previously (114). Alltogether 44 patients developed ischemic cardiovascular events (composite end-points of death, myocardial infarction, recurrent unstable angina, or stroke) during the follow-up (555.4±21.2 days): unstable angina pectoris (n = 14), non Q-wave acute myocardial infarction (n = 14), Q-wave acute myocardial infarction (n = 5), cerebral infarction (n = 4), death (n = 7). Elective bypass-operation (coronary artery bypass graft, n = 36) or balloon angioplasty (percutaneuous coronary intervention, n = 33) were not considered as end-points. Blood samples were taken within 48 hours of hospitalization (visit 1), at 1 week (visit 2), at 3 months (visit 3), and at 1 year (visit 4) after hospital admission. At the first visit, 36 patients (24.32%) had a documented previous event of myocardial infarction.

8. METHODS

Laboratory methods used in the study are summeruzed in Table 8.

Table 8. Laboratory methods used in the present study.

Laboratory work	Method	Study
DNA isolations		I
ACS patients	Puregen Kit	
Age- and sex-matched controls	Salting out	
HLA-A, B, and -DRB1 typing		I
Heart Tx recipients	Phenotype (Complement mediated lymphcytotoxicity test)	
ACS patients	Genotype (HLA-ABDR SSPtray)	
Age- and sex-matched controls	Genotype (HLA-ABDR SSPtray)	
Four LTA SNPs typing[1]		
ACS patients	Genotype (Allele specific PCR)	
Age- and sex-matched controls	Genotype (Allele specific PCR)	
Complement C4A and C4B allotyping		I, II, III, IV
ACS patients	Electrophoresis and immunofixation	
Age- and sex-matched controls	Electrophoresis and immunofixation	
Measurement of C3 and C4 concentrations		III, IV
ACS patients	Blood/serum nephelometry	
Age- and sex-matched controls	Blood/serum nephelometry	
Measurement of *C. penumoniae* markers		II
ACS patients		
C. pneumoniae IgA, IgG	Microimmunofluorescence	
C. pneumoniae immune complex	Polyethylene glycol precipitation	
Age- and sex-matched controls		
C. pneumoniae IgA, IgG	Microimmunofluorescence	
C. pneumoniae immune complex	Polyethylene glycol precipitation	
Measurement of cotinine		II, IV
Age- and sex-matched controls	Gas chromatography	
Sequencing of LTA and determination of SNPs and sequence analysis	ABI 3100 Genetic Analyzer, ProSeq v 2.91 software	I, III
ACS patients		
Oral status		III
ACS patients	Orthopanthomography	
Periodontal bacterial culture from saliva		III
ACS patients		
P. gingivalis	Culture	
A. actinomycetemcomitans	Culture	
Periodontal bacterial PCR from saliva		III
ACS patients		
P. gingivalis	Species specific PCR	
A. actinomycetemcomitans	Species specific PCR	
Serum analysis for periodontal pathogens		III
ACS patients		
P. gingivalis IgA, IgG	Multiserotype ELISA	
A. actinomycetemcomitans IgA, IgG	Multiserotype ELISA	

[1] We used four LTA markers [+253(a/g) in intron 1; +496(C/T) in exon 2; +633(c/g) in intron 2; and +724(C/A) in exon 3] (168, 294) (Figure 28) for the genomic typing and the haplotype analysis of patients with ACS and controls. See Appendix for abbreviations.

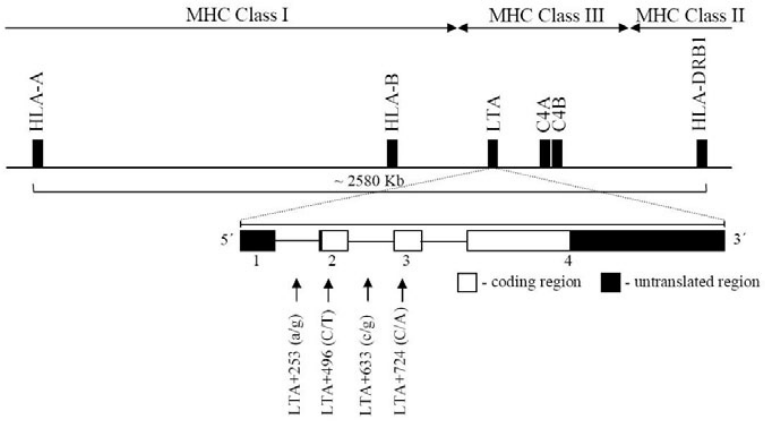


Figure 28. Approximate localization of studied genetic markers. See Appendix for abbreviations. Modified from: Study I.

Quantitative measurements of total concentration of lipopolysaccharide in serum was done using the Limulus Amoebocyte Lysate assay with a chromogenic substrate (HyCult Biotechnology B.V., The Netherlands) on diluted (1:5, v/v, in endotoxin-free water) samples.

Statistical methods used in the study are summeruzed in Table 9.

Table 9. Statistical methods used in the present study.

Statistical Methods	Study
Inference of MHC haplotype by PHASE v. 2.02 software	I
Sample size and power calculation	I, III
Linkage disequilibrium test by ARLEQUIN v 2.000 software	I
Chi-Square test or Fisher's exact test	I,II,III,IV
Quartiles of C3/C4 ratio	IV
Cut-off value of C3/C4 ratio	IV
Receiver operating characteristic curve	IV
Multiple logistic regression analysis	I,II,III,IV
Kaplan-Meier survival analysis	IV
Cox multivariate logistic regression survival analysis	IV
ANOVA repeated measure	IV
Student-T test	III, IV
Geometric mean value test	II, III
Visualization of 1TNR Protein Data Bank file	III

ANOVA = Analysis of variance, MHC = Major histocompatibility complex.

Based on the results received from the HTx study (see Section 10.1 for details), the HLA-DRB1*01 was considered as a predisposing allele. The Finn90 cohort (275) provides the HLA-DRB1*01 allele frequency of 0.13. The power calculations for ACS study showed (with 80% power, 2 sides $\alpha = 0.05$) a sample size of 100 cases (number of chromosomes = 200) and 74 controls (number of chromosomes = 148) to be enough to detect an odds ratio of 2.2 (Figure 29).

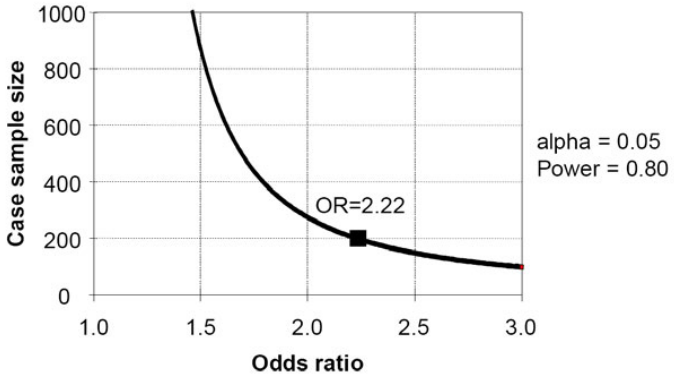


Figure 29. Power calculation for coronary artery disease.
OR = Odds ratio.

We have used an antibody titer of ≥40 for IgA, ≥128 for IgG, and ≥2 for IC as elevated marker of *C. pneumoniae* infection, suggesting chronic infection. Antibodies are only an indirect evidence of chronic *Chlamydial* infection, and the antibody levels will not stay high without continuous infection (81, 83).

The 1TNR protein data bank file was visualized with the Accelrys Discovery Studio v1.7 software (http://www.accelrys.com/products/dstudio/). The power calculations and graphs were plotted (295) using the PS power and sample-size software (296). To infer the haplotype from the study subjects, the popular (297, 298) software PHASE V. 2.02 (299, 300) was used. The Arlequin 2.000 software (301) was used to achieve the linkage disequilibrium values. For all other statistical analyses, we used SPSS 12.0.1 statistical package (SPSS Inc., Chicago, IL).

9. ETHICAL CONSIDERATIONS

All patients and controls gave a written informed consent. The study protocols for HTx and ACS studies were approved by the Ethics Committees of Department of Medicine, Hospital District of Helsinki and Uusimaa, and for the controls study also by the Finnish Red Cross Blood Transfusion Service.

Patients with ACS (Figure 27) were collected for a placebo-controlled clarithomycin study on ACS (114), where the MHC genes on these patients were evaluated. The HTx recipients and age- and sex-matched controls (Figure 27) were newly collected for this study.

10. RESULTS

10.1. Study I

Both studies, the HTx and ACS, consistently showed HLA-A3– B35– DR1 -related haplotypes as risks for CAD (HTx study, OR = 2.10, 95% CI = 1.04 to 4.23, P = 0.03; and ACS study, OR = 5.14, 95% CI = 1.75 to 15.12, P = 0.001). In the ACS study, the extended MHC haplotype of HLA-A3– B35– DR1, with the deficiency of C4B protein and the presence of LTA*011 allele (LTA+253a– LTA+496C– LTA+633c– LTA+724C), was associated with CAD (OR = 10.22, 95% CI = 1.32 to 79.02, P = 0.0063), whereas the haplotypic combination of HLA-DRB1*01 and LTA+724C showed the strongest P-value (OR = 3.34, 95% CI = 1.61 to 6.94, P = 0.0007). Markers in the extended haplotype were in strong linkage disequlibrium. On the other hand, HLA-B*07 and HLA-DRB1*15 without the deficiencies of C4 protein and with the intronic LTA markers of LTA +253a and LTA+633g were shown as protective markers for CAD (OR = 0.24, 95% CI = 0.06 to 0.88, P = 0.02), whereas the haplotype from the class III region (LTA+253a− LTA+633g− C4A3− C4B1) was highly significant (OR = 0.36, 95% CI = 0.22 to 0.57, P = 0.00001). These protective markers showed no association with the clinical parameters.

In the univariate analysis, HLA-DR1 was associated with CAD in both studies (HTx study, OR = 2.37, 95% CI = 1.33 to 4.25, P = 0.003; and ACS study, OR = 2.36, 95% CI = 1.25 to 4.44, P = 0.007). The number of enrolled subjects was high enough to detect the 80% statistical power with odds ratio of 2.2 for HLA-DR1 allele (Figure 29). When we performed the step-wise multiple logistic regression analysis separately in both studies, HLA-DR1 remained as a significant risk for CAD (HTx study, OR = 3.46, 95% CI = 1.74-6.91, P = 0.0004; and ACS study, OR = 2.34, 95% CI = 1.23 to 4.43, P = 0.009). HLA-DR1 was further associated with several other CAD risk factors, e.g., diabetes mellitus (OR = 5.08, 95% CI = 1.43 to 18.06, P = 0.012), increased serum LDL (OR = 5.32, 95% CI = 1.64 to 17.26, P = 0.005), smoking habit (OR = 3.13, 95% CI = 1.09 to 9.03, P = 0.035), and C4B*Q0 (OR = 12.95, 95% CI = 4.65-35.04, P = 0.0000009). Altogether, HLA-A3– B35– DR1 haplotype is the risk haplotype for CAD, and HLA-DR1 is the strongly associated marker.

10.2. Study II

Patients with CAD had elevated *C. pneumoniae* infection markers when compared with age- and sex-matched controls (OR = 1.85, 95% CI = 1.00 to 3.42, P = 0.049). The main finding in this study was that HLA-B*35 or -related haplotypes associated with *C. pneumoniae* infection markers in patients with CAD.

Among the patients with CAD, HLA-B*35 (OR = 7.88, 95% CI = 2.44 to 25.43, P = 0.0006) was the strongest risk gene for *C. pneumoniae* infection in the step-wise multiple logistic regression analysis. Similarly, in the univariate analysis, HLA-B*35 or -related haplotypes (HLA-A*03− B*35 or HLA-A*03−

B*35− LTA+724C) were also associated (HLA-B*35: OR = 2.75, 95% CI = 1.14 to 6.64, P = 0.02; HLA-A*03− B*35: OR = 4.27, 95% CI = 1.64 to 11.12, P = 0.002; or HLA-A*03− B*35− LTA+724C: OR = 3.19, 95% CI = 1.18 to 8.64, P = 0.02). Eighty-one percent of patients with HLA-A*03− B*35, and HLA-A*03− B*35− LTA+724C haplotype had *C. pneumoniae* infection markers, whereas patients with HLA-DRB1*01 had about 55%. HLA-A*03− B*35 (P = 0.044) or HLA-A*03− B*35− LTA+724C (P = 0.048) haplotypes showed significantly elevated *C. pneumoniae* infection markers, when compared with HLA-DRB1*01. *C. pneumoniae* infection markers were further associated with males having the HLA-A*03− B*35 (P = 0.008) or HLA-A*03− B*35− LTA+724C (P = 0.02) haplotypes as well as with smokers with these haplotypes (P = 0.001, or P = 0.009, respectively).

Among controls, no correlation between *C. pneumoniae* markers and the MHC genes, smoking status, or gender were found.

10.3. Study III

Occurrence of *P. gingivalis* in saliva was more frequent in patients with periodontitis (OR = 4.74; 95% CI = 1.54 to 17.32; P = 0.002), when compared with non-periodontitis patients. Similary, serum antibodies of the pathogen were also elevated (IgG: P = 0.0073, and IgA: P = 0.048). The major finding of this study was that LTA+496C associated with periodontitis in patients with CAD.

In the forward step-wise multiple logistic regression analysis, LTA+496C (OR = 10.87, 95% CI = 3.23 to 36.6, P = 0.00012), and elevated serum IgA antibodies of *P. gingivalis* (OR = 1.57, 95% CI = 1.05 to 2.30; P = 0.026) were associated with periodontitis, when comparison between periodontitis vs non-periodontitis was made and the following covariates or confounding factors were included: MHC gene markers [HLA-A, HLA-B, LTA+253(a/g), LTA+496(C/T), LTA+633(c/g), LTA+724(C/A), C4A, C4B, and HLA-DRB1], periodontal bacterial markers (occurrence of *A. actinomycetemcomitans* and *P. gingivalis* in saliva, and IgA and IgG antibody levels against these pathogens in serum), and periodontal risk factors (age, sex, smoking habits, and diabetes mellitus), lipids (total cholesterol, HDL-cholesterol, LDL-cholesterol levels in serum, and use of statins), and others (hs-CRP, hypertension arterialis, and body mass index).

In the univariate analyses, patients with periodontitis had increased frequencies of LTA+496C (CC vs TT: OR = 8.46, 95% CI = 1.78 to 40.29, P = 0.0026; and CC + CT vs TT: OR = 5.29, 95% CI = 2.07 to 13.51, P = 0.00027), LTA+633c (cc + cg vs gg: OR = 3.58, 95% CI = 1.13 to 11.34, P = 0.024), HLA-DRB1*01 (OR = 3.26, 95% CI = 1.12 to 9.46, P = 0.025), and HLA-B*35 (OR = 3.10, 95% CI = 1.06 to 8.95, P = 0.033) when compared to those without periodontitis. Among the tested alleles, only LTA+496C showed 80% statistical power (Figure 30).

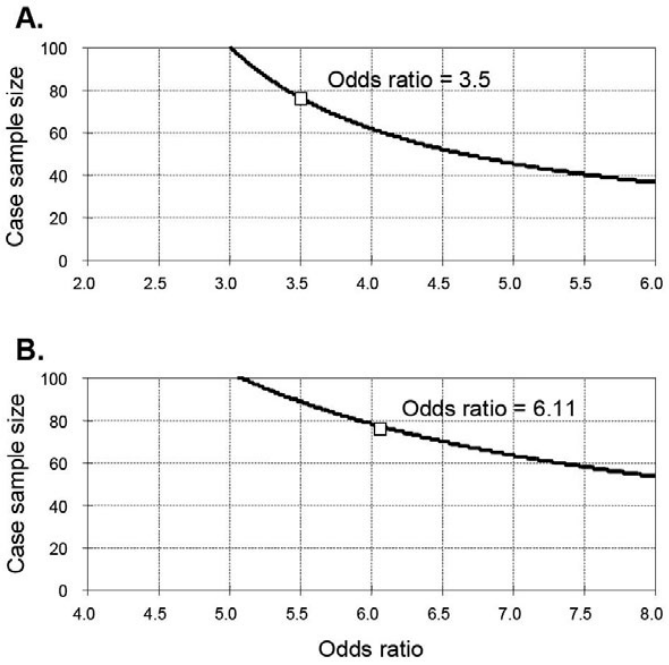


Figure 30. Power calculations for periodontitis. Odds ratio of 3.5 for LTA+496C (**A**) and 6.11 for LTA+496CC (**B**) would be enough to detect 80% power with two sides α = 0.05 for sample size for 76 periodontitis as cases.

Patients having HLA-DRB1*01 had elevated serum antibodies of *P. gingivalis* (IgA: P = 0.041 and IgG: P = 0.028) and serum total-lipopolysaccharide (P = 0.038). Increasing quartiles of total-lipopolysaccharide associated with increasing serum complement C3 and C4 concentrations (Figure 31).

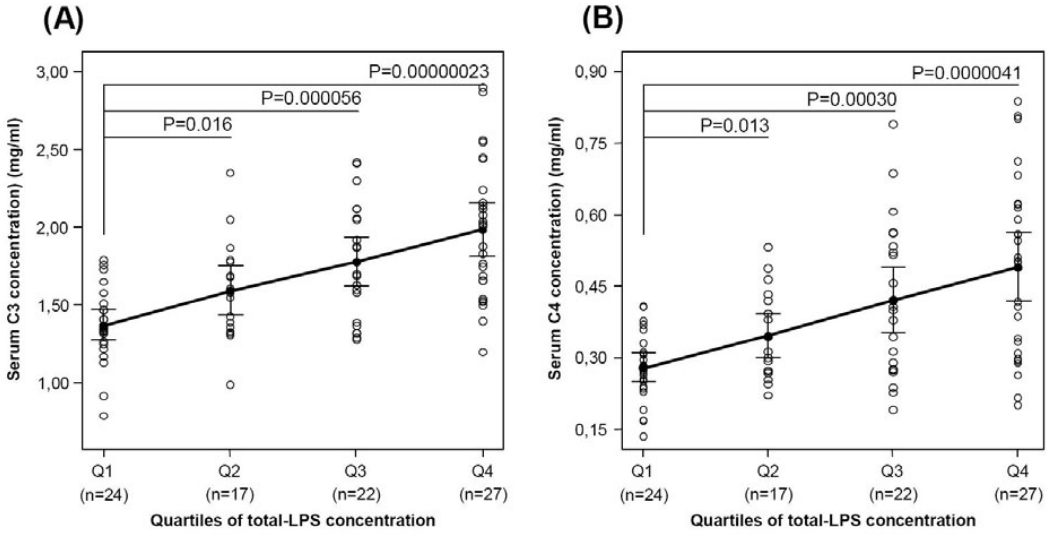


Figure 31. Associations of total-lipopolysaccharide quartiles and complement component C3 (A) and C4 (B) proteins. C3 = Complement component C3, C4 = Complement component C4, LPS = Lipopolysaccharide, Q = Quartile.

10.4. Study IV

Serum C3 and C4 concentrations had a strong positive correlation in patients with CAD (r = 0.77, P = <0.00001) (Figure 32). Of the 148 patients recruited, 44 patients met ≥1 end-point (unstable angina pectoris, n = 14; myocardial infarction, n = 19; cerebral infarction, n = 4; or death, n = 7). Patients with end-point had trendlike decreased serum C4 concentrations (repeated measure ANOVA, P = 0.06) and borderline increased serum C3 concentrations (Student T-test, P = 0.057), when compared alone to patients without end-point. The major finding of this study was that the serum complement C3/C4 ratio as a novel marker was associated with recurrent cardiovascular events in patients with CAD.

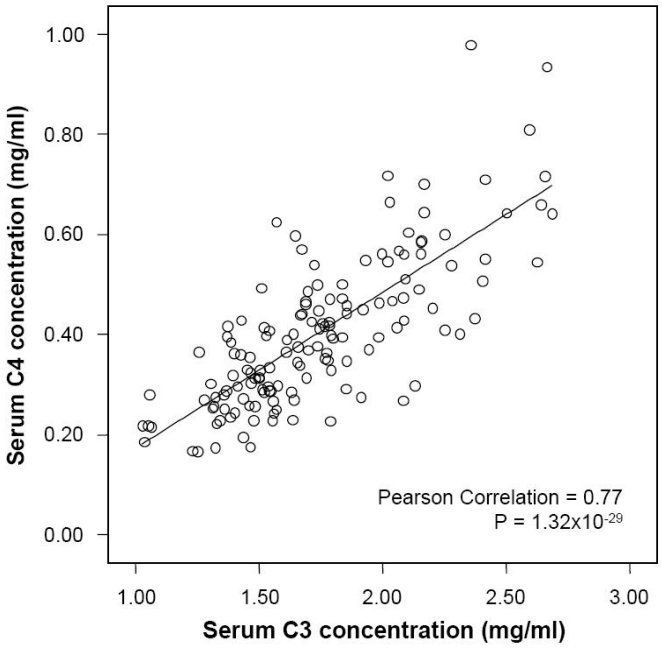


Figure 32. Correlation of serum C3 and C4 concentrations.

Patients with an end-point showed increased levels of C3/C4 ratio (repeated measures ANOVA, P = 0.007), when compared to patients without one. Along with the presence of diabetes mellitus (OR = 2.12, 95% CI = 1.06 to 4.24, P = 0.03), the increased C3/C4 ratio (OR = 1.33, 95% CI = 1.08 to 1.63, P = 0.007) was regarded as a novel risk factor for recurrent cardiovascular end-points in the Cox multiple logistic regression survival analysis, when wide ranges of covariates were included: age, gender, blood pressure, diabetes mellitus, body mass index, smoking, total cholesterol, high density lipoprotein cholesterol, LDL, triglyceride, high sensitive C-reactive protein, Canadian Cardiac Society classes, pro-brain natriuretic peptide, troponin T, acetylsalicylic acid, beta-adrenergic blockers, antiotensin-converting enzyme or angiotensin inhibitor, and statins, C3/C4 ratio, pulse rate, glucose, leukocyte count, thrombocyte count, hemoglobin, glycohemoglobin, creatinine, liver enzymes, and thromboplastin percentage.

We took 4.53 as a cut-off value in the C3/C4 ratio for end-points using the group statistics, and analysed in the receiver operating characteristic curve. It showed >60% of specificity and sensitivity for the cut-off value. Patients having higher than the cut-off value were associated with increased end-points (OR = 2.25, 95% CI = 1.09 to 4.65, P = 0.028) and worst survival (Log Rank, P = 0.014, survival curve not shown). Similarly, patients having the highest quartile of C3/C4 ratio had also increased end-points (OR = 3.04, 95% CI = 1.27 to 7.29, P = 0.01; worst survival: Log Rank, P = 0.005). Both higher than the cut-off value (data not shown) and the highest quartile of C3/C4 ratio showed increased C3 and decreased C4 serum levels in the regression statistics. One unit change in C4 concentration generated a 1.7-fold change in C3 concentration in the highest quartile and a 1.24-fold change in the medium quartile compared with the change in the low quartiles.

Patients with C4*Q0 alleles (presence of either C4A*Q0 or C4B*Q0) had a high C3/C4 ratio (repeated measures ANOVA, P = 0.0014) and decreased serum C4 concentrations (repeated measures ANOVA; P = 0.0018), when compared to patients without C4*Q0. The C4*Q0 and C4A*Q0 alleles were increased in patients having the highest quartile (C4*Q0: OR = 5.70, 95% CI = 1.50 to 21.66, P = 0.006; and C4A*Q0: OR = 3.17, 95% CI = 1.07 to 9.41, P = 0.03) and higher than cut-off value (C4*Q0: OR = 2.73, 95% CI = 1.12 to 6.65, P = 0.025; and C4A*Q0: OR = 2.90, 95% CI = 1.12 to 7.47, P = 0.025). In addition, patients with C4A*Q0 allele had an increased number of end-points (OR = 2.94, 95% CI = 1.17 to 7.40, P = 0.02) and worst survival (Log Rank, P = 0.012).

11. Discussion

11.1. Study I

We evaluated several MHC genes in two independent patient cohorts, the HTx and ACS study (Figure 27). Both studies consistently showed HLA-A3– B35– DR1 and -related haplotype as risks for CAD. This haplotype is known as the 35 ancestral haplotype and is more frequent in Finland than in other European countries (Finland = 7.8% *vs* other European countries = 1.3%) (275).

The ACS study showed not only the susceptible but also a protective haplotype for CAD. The susceptibility haplotype consisted of HLA-A3– B35– DR1, with the deficiency of C4B protein and the presence of the LTA*011 allele (LTA+253a– LTA+496C– LTA+633c– LTA+724C). The protective haplotype included HLA-B*07 and HLA-DRB1*15 with functional C4 gene and with the intronic LTA markers of LTA +253a and LTA+633g. The HLA-A3– B35– DR1 haplotype was significantly increased in patients with CAD in both studies, and the HLA-DR1 allele alone was associated with CAD both in univariate and multiple logistic regression analyses including the studied genetic loci and clinical predictors for CAD. The HLA-DR1 was further associated with CAD risk factors, e.g., diabetes mellitus, increased serum LDL, smoking, and C4B*Q0. This shows that the involvement of HLA-DRB1*01 in CAD pathogenesis is very wide.

Increased levels of LDL, presence of diabetes mellitus and smoking are traditional risks for CAD, while presence of C4B*Q0 (9, 302) is an emerging risk for CAD. Recently it has been shown from the Icelandic population that C4B*Q0 is associated with mortality after acute myocardial infarction (10). The HLA-DRB1*01 was associated with diabetes mellitus, and CAD protective haplotype included HLA-DRB1*15. This is in agreement with the fact that diabetes mellitus is an established risk factor for CAD. Some studies indicate that HLA-DR1 is more prevalent in diabetes, also in early diabetic retinopathy, whereas HLA-DR15 is less prevalent in diabetes (303-305). In addition, Langerhans islet cell antigen 69 (ICA69), which is recognized by T cells of diabetes patients, is strongly bound to HLA-DR1 (306). In the present study, the frequency of C4B*Q0 showed only a trend-like increase in patients with CAD when compared to controls (OR = 1.62, 95% CI = 0.93 to 2.82, P = 0.084). However, smokers carrying C4B*Q0 along with HLA-DRB1*01 were significantly associated with CAD.

The LTA+253g allele is one of the risk alleles for myocardial infarction (168). In agreement with this, we also showed that LTA+253g (OR = 1.79, 95% CI = 1.11 to 2.89, P = 0.018), and LTA+633c (OR = 2.78, 95% CI = 1.39 to 5.56, P = 0.004) were significant risks for CAD in the step-wise multiple logistic regression analysis, when HLA genes were excluded. This result may suggest that LTA+253g and LTA+633c might have independent or individual effects on CAD, but HLA-DRB1*01 seemed more important.

The present studies included two different groups of patients with CAD. The HTx study consisted of patients with the most severe form of stable angina, and the patients with ACS with unstable angina. These two separate groups were enrolled to examine whether stable and unstable angina share similar genetics. The pathophysiology of unstable angina differs from that of stable angina. The severity of inflammation in unstable angina is stronger than in stable angina (38); however, the patients with stable angina may have undergone periods of unstable angina. CAD is an inflammatory disease (1), and for the present study we considered the MHC region as a candidate region for inflammatory disease. This study provides evidence that both stable and unstable angina may follow the same genetic risk factors: the HLA-A3– B35– DR1 -related haplotypes.

There are only few studies available showing the direct or indirect significance of the MHC region on CAD (e.g., MHC2TA, LTA, and LGALS2 in Table 3; and, e.g., C4A and C4B null alleles in Table 4). These associated genes might be inherited in linkage disequilibrium with each other or with HLA classical genes forming an ancestral MHC haplotype with different lengths. The haplotype may have favorable effects on several biological effects and therefore have been frequent under positive selection pressure. However, no such study is available. We took the advantage of using multiple markers, which included most of the previously studied MHC gene markers. The present study showed for the first time that both the susceptibility (HLA-A3– B35– DR1, 35 ancestral haplotype) and protective (HLA-B*07 and HLA-DRB1*15 -related haplotype) haplotype for CAD exist in the MHC region. However, the association of HLA-DR1 with CAD had over 80% statistical power.

Briefly, the Study I showed that specific MHC genes can partially explain the development of CAD in patients possessing several risk factors, e.g., diabetes mellitus, increased serum LDL, smoking habit, and C4B*Q0. In conclusion, the human MHC region harbors both susceptibility and protective haplotypes for CAD.

11.2. Study II

In agreement with previous reports (78, 79, 82, 83), this study also showed that patients with CAD had significantly elevated *C. pneumoniae* markers when compared to age- and sex-matched controls. HLA-B*35 or -related haplotypes were associated with *C. pneumoniae* infection in patients with CAD.

C. pneumoniae is one of the important pathogenes that associate with CAD. Here, we used the presence of *C. pneumoniae* IgA (titer, ≥40), and IgG (titer, ≥128) antibodies or IC-bound IgG antibodies (titer, ≥2) as serological markers, suggesting chronic *C. pneumoniae* infection (81, 83, 307). However, these antibodies give only indirect evidence of chronic chlamydial infection, as their levels do not stay high without continuous infection (81, 83).

HLA-B*35 was associated with *C. pneumoniae* infection markers both in univariate and step-wise multiple logistic regression analyses. Univariate analyses further showed that not only the HLA-B*35 allele, but also the HLA-B*35 -related haplotypes (HLA-A*03− B*35 or HLA-A*03− B*35− LTA+724C) were associated with elevated *C. pneumoniae* infection markers. The HLA-B*35 and -DRB1*01 -related haplotypes are risk factors for CAD (Study I), and *C. pneumoniae* is one of the most important risk pathogens (11). When the presence of HLA-B*35 -related haplotypes was compared with HLA-DRB1*01, elevated *C. pneumoniae* infection markers were significantly associated with the presence of HLA-B*35 -related haplotypes. This shows the importance of HLA-B*35 -specific MHC class I molecules in relation to *C. pneumoniae* infection and CAD. Markers of *C. pneumoniae* infection were further associated with males and smokers having the above haplotypes. The male gender and smoking habits have long been established risk factors for CAD, and they affect the incidence or persistence of *C. pneumoniae* infections (15, 308), too. However, among the age- and sex-matched controls, no correlations were found between *C. pneumoniae* infection markers and MHC genes, smoking status, or gender. This suggests that an established chronic *C. pneumoniae* infection in patients with CAD is the result of multiple factors, and HLA-B*35 -related haplotypes are among them.

The HLA-B*35 allele is one of the most frequent HLA alleles in the Finnish population, with the highest percentage in the eastern parts (309). Similarly, the incidence of CAD (310, 311) and the prevalence of *C. pneumoniae* markers (312) are elevated in eastern Finland. This suggests an accessible association between CAD, *C. pneumoniae* and HLA-B*35.

C. pneumoniae infected cells can induce several genes, e.g., vasoconstrictor endothelin-1 (313) and genes involved in apoptosis (314). Also, HLA-B*35 has been shown to influence the apoptosis rate (315), and the upreguation of endothelin-1 (316). MHC class I molecule-specific peptides, especially HLA-B35, are of a wide range, and can bind atypically-long CD8 T cell target peptides (317-319). However, no such atypically-long peptides have been reported as yet in *C. trachomatis*, but the HLA-B35-restricted peptides (major outer membrane protein and heat shock 60 kDa protein) are known to result in CD8 T cell responses (320). In the present study, the HLA-B*35 -specific haplotype which showed increased *C. pneumoniae* markers included also the LTA+724C allele. LTA can reduce the growth of *C. pneumoniae* (321). No data are available whether LTA+724C influences the expression of LTA, but LTA+252(a/g) (168) can enhance the transcriptional level of LTA.

No studies are available in relation to MHC genes and *C. pneumoniae* in a normal population. In the CAD setting, very few reports are available. Dahlén GH et al (189) studied MHC class II genes and showed an association between *C. pneumoniae*, high Lp(a) level and HLA-DRB1*03 and -DRB1*13; however, the association was not confirmed later (190). We studied several genetic markers spanning the whole MHC region. The present study showed for the first time that the HLA-B*35 (class I gene and telomeric part of the

CAD susceptibility haplotype, Study I) explains the *C. pneumoniae* infection in patients with CAD, but not class II genes (e.g., HLA-DRB1*01).

Our results in Study II may indicate that HLA-B35 presents *C. pneumoniae* peptides, and may further provide an environment that predetermines the establishment of persistent *C. pneumoniae* infection. We found an association between the markers of *C. pneumoniae* infection and HLA-B*35 -related genes, which was even more pronounced in males and smokers having these genes. In conclusion, the HLA-B*35 positive haplotypes confer *C. pneumoniae* -related risk for CAD.

11.3. Study III

CAD patients with periodontitis had increased occurrence of *P. gingivalis* in saliva, and elevated serum antibodies of the pathogen. LTA+496C strongly associated with periodontitis, whereas HLA-DRB1*01 associated with elevated serum antibodies of *P. gingivalis*.

LTA+496C, one of the markers of the HLA-A3– B35– DR1 haplotype, was associated with CAD patients with periodontitis in both univariate and step-wise multiple logistic regression analyses. The association between LTA+496C and periodontitis had 80% statistical power. In univariate analyses, the frequencies of other MHC genes (LTA+633c, HLA-DRB1*01, and HLA-B*35) were also significantly increased in periodontitis, but the elevated serum antibodies of *P. gingivalis* and elevated levels of serum total-lipopolysaccharide were associated only with HLA-DRB1*01. The LTA+496C allele is in strong linkage with LTA+633c, HLA-DRB1*01, and HLA-B*35 (Study I). Total-lipopolysaccharide strongly correlated with serum complement C3 and C4 concentrations, suggesting enhanced immune responses in the presence of HLA-DRB1*01. Briefly, LTA+496C and HLA-DRB1*01 might be links between periodontitis and CAD.

The LTA gene is associated with periodontitis (322, 323), but LTA+496(C/T) has not been studied yet. We showed LTA+496C as a strong risk for periodontitis in patients with CAD. At the sequencing level, the LTA+496(C/T) variation was found to reside in exon 2 substituting 13^{th} Arg13Cys amino acid, belonging to the signal peptide region. It is not known whether the 13^{th} amino acid substitution affects inter- or intramolecular bonding. However, cysteine-specific disulphide bonds are crucial in defining the structure of many proteins through inter- and intra-molecular bondings (324); on the other hand, guanidine moieties (guanidinium group) on arginine's long side chains are responsible for the majority of arginine noncovalent interactions (325). The modeling of the signal peptide region showed that the Arg13Cys influences the hydrophilic and electrostatic properties of the region. Thus, it is quite possible that the amino acid substitution (Cys_{13} -> Arg_{13}) may influence on folding and interaction with target molecules. In Figure 33, the region which is shown as non-underlined but belongs neither to signal peptide region nor to TNF domain, contains the Thr_{41} amino acid (encircled amino acid in Figure 33), which is known as the site of O-

glycosylation (326, 327). Thus, this O-glycosylation site migh have biological importance during the protein folding process. We visualized the 1TNR Protein Data Bank file, and analyzed the sequence. The yellow part in the ligand molecule (molecule with red solid ribbon in Figure 33) corresponds to the amino acids (amino acids marked with triangles in Figure 33) interacting with its receptor with 5 ångstroms radius.

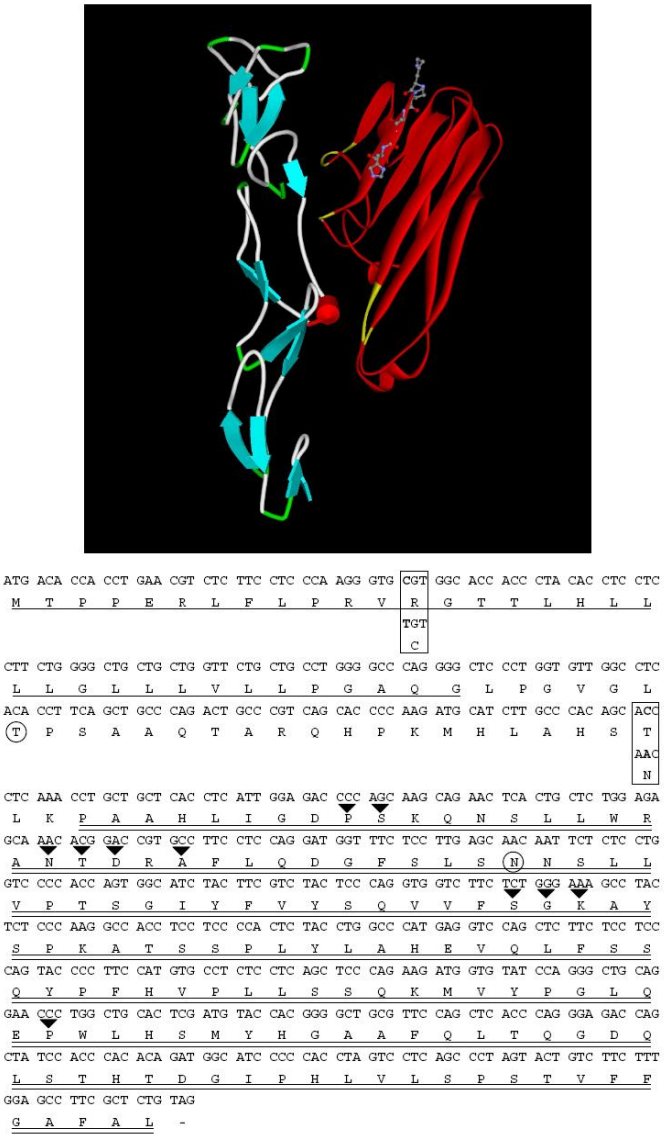


Figure 33. Visualization of 1TNR protein data bank file and lymphotoxin alpha amino acid and coding sequence. The intronic regions are not shown. The studied exonic SNPs [LTA+496(C/T) and +724(C/A)] are bolded and rectangled with their corresponding variations. Amino acids belonging to the signal peptide are single-underlined, the TNF domain double-underlined. The LTA molecule (red solid ribbon) corresponds only with the TNF-domain and the receptor molecule (schematic with alpha and beta structure) with the tumor necrosis factor receptor domain of TNFR receptor (data not shown). Amino acids indicated with triangles (or yellow part in the ligand molecule) are those amino acids which interacted with its receptor molecule with 5 ångstroms radius. Site of O-glycosylation (Thr_{41}) and N-glycosylations (Asn_{96}) are encircled. LTA = Lymphotoxin alpha, SNP = Single nucleotide sequence, TNF = Tumor necrosis factor, TNFR = Tumor necrosis factor receptor superfamily. Modified from: Study IV.

Infections which are related to periodontal disease are also risks for CAD (132, 160). For example, *P. gingivalis* is a major periodontal pathogen (328, 329) and can accelerate atherosclerosis (153), and invade

periodontal tissues and vascular endothelium (152, 330). High serum antibodies of *P. gingivalis* associate with increased risk for stroke (146, 147), and myocardial infarction (143-145). Thus, the association between HLA-DRB1*01 and radiological evidence of periodontitis, and bacteriological and serological evidence of *P. gingivalis* may serve as an explanation that the HLA-DRB1*01 is one of the shared factors between the *P. gingivalis*, periodontitis, and CAD.

Several studies have been performed investigating MHC genes (especially classical HLA genes) for periodontitis (331, 332). In the replication studies, the results were somewhat confilicting. This is perharps due to the complex nature of the HLA genes, or the insufficient statistical power. Genetic variation in LTA gene from the MHC class III region seems to be of great interest, perhaps due to its association with myocardial infarction, or because it is a proinflammatory cytokine with a variety of functions. Two studies indicate a link between periodontitis and the LTA gene (322, 323). We studied four different functionally important single nucleotide polymorphisms in the LTA gene, including several other genetic markers from the MHC region. The present study shows for the first time that LTA+496C (class III gene and central part of the CAD susceptibility haplotye, Study I) strongly associates with periodontitis, and HLA-DRB1*01 (class II gene and centromeric part of the CAD susceptibility haplotye, Study I) with *P. gingivalis* infection.

In conclusion, Study III suggests that LTA+496C predisposes patients with CAD to periodontitis, while other MHC-genes (e.g., LTA+633c, HLA-B*35, and HLA-DRB1*01) also play important roles, probably due to their tight linkage with LTA+496C or due to the interaction with *P. gingivalis* (e.g., HLA-DR1).

11.4. Study IV

Complement C3/C4 ratio was a novel concept in the complement biology and was associated with the recurrent cardiovascular end-points. The increased level of C3/C4 ratio was a better predictor than the serum C3 and C4 concentrations. The relative increase of serum C3 protein and decrease in C4 protein could explain the increased levels of the C3/C4 ratio.

Chronic inflammation is one of the main underlying factors in CAD (1). An increased serum C3 concentration (27-29) and the presence of C4 null alleles (9, 10) are risk factors for CAD. When C4 quantiative null alleles are present, the level of C4 synthesis may not match the need, partially explaining the deficiency of C4 proteins (30, 31). Patients with end-points showed marginally increased levels of serum C3, and marginally decreased C4 concentration. Thus, we hypothesized that the presence of both (increased serum C3 and decreased C4 concentration) serves as an index of enhanced vascular inflammation. Small changes in C3 and C4 concentrations to the opposite directions are responsible for the change in the C3/C4 ratio, and this could be a better predictor than the mere C3 and C4 concentrations. To prove this hypothesis, we used the C3/C4 ratio as a novel concept in complement biology (Figure 34), and showed the increased

levels of the C3/C4 ratio as a novel risk factor for cardiovascular end-points with the two-way ANOVA and multivariate logistic regression analysis. The C3/C4 ratio is influenced greatly by the decreased level of C4, and the decreased level of C4 is due to low C4 gene dosage (e.g., presence of C4 null alleles) or other factors (e.g., complement activations and immune complexes). In addition, patients having the higher than cut-off value and the highest quartile of the C3/C4 ratio were also associated with the end-points.

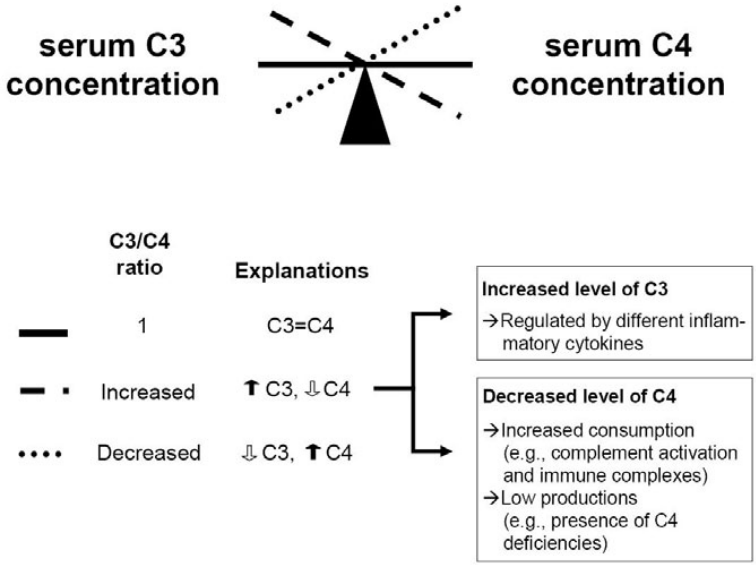


Figure 34. Serum complement C3/C4 ratio hypothesis. Both the increased serum level of C3 and decreased serum level of C4 explain the increased C3/C4 ratio. Low production, increased consumption of C4, and increased expression of C3 regulated by different inflammatory cytokines modify the increased C3/C4 ratio.

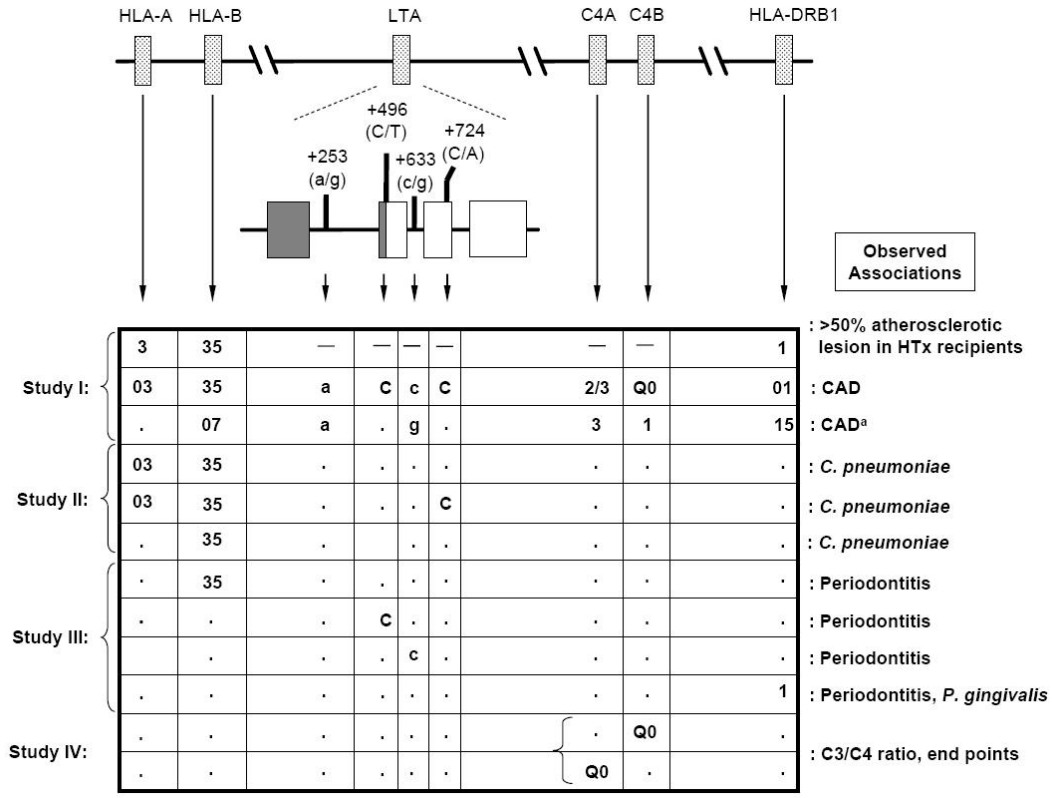
Recent studies show that serum complement C3 and C4 protein levels are independent risks for hypertension, stroke, and myocardial infarction (333-335). The C3 and C4 levels further correlate with cardiovascular risk factors (e.g., blood pressure, body mass index, lipids, and C -reactive protein), also with intercellular adhesion molecule-1 (335, 336). The C4 mRNA and protein can be induced by IFN-γ, but can be inhibited by IL-1B (337, 338). C4 levels are also influenced by the number of C4 genes (30, 31). In agreement with this, we showed that C4*Q0 alleles (presence of either of C4A*Q0 or C4B*Q0) were associated with an increased C3/C4 ratio, and a decreased level of serum C4 concentration. C4*Q0 and C4A*Q0 alleles were also more frequent in patients having higher than cutt-off value and the highest quartile of C3/C4 ratio. The C4A*Q0 allele also significantly associated with increased end-points (OR = 2.94, 95% CI = 1.17 to 7.40, P = 0.02), and worst survival (Log Rank, P = 0.012, survival curve not shown). Furthermore, it has recently been shown in the Icelandic population that C4B*Q0 is associated with mortality after acute myocardial infarction (10). Thus, it is quite possible that the decreased levels of serum C4 concentration might be a risk factor, but the optimum level of C4 might prevent cardiovascular events. However, no increased risk for cardiovascular complications has been described as yet in patients with complete C4 deficiency. There is some information that defective function of the 21-hydroxilase gene next to the C4B gene might have an effect on the decreased survival rate of the low C4B copy number carriers (10).

Ischemic cardiovascular risk factors, increase in serum C3 levels (27-29) and the presence of C4 null alleles (9, 10), can influence the C3/C4 ratio. This is the first study to use this novel concept of C3/C4 ratio and to show it as a predictive marker for ischemic cardiovascular end-points.

Briefly, in Study IV we showed that the low production, increased consumption of C4, and increased expression of C3 regulated by different inflammatory cytokines modify the C3/C4 ratio (Figure 34). In conclusion, increased serum C3/C4 ratio is a new risk marker for recurrent cardiovascular events in patients with CAD. The relative increase in serum C3 protein and decrease in C4 protein could explain changes in the C3/C4 ratio.

12. CONCLUSIONS

The main results of this study are summerized in Figure 35.



	3	35	—	—	—	—	—	—	1
Study I:	03	35	a	C	c	C	2/3	Q0	01
	.	07	a	.	g	.	3	1	15
Study II:	03	35	.	.	.	.	.	.	.
	03	35	.	.	.	C	.	.	.
	.	35	.		.	.	.	.	.
Study III:	.	35	.	.	.	.	.	.	.
	.	.	.	C	.	.	.	.	.
		.	.	.	c	.	.	.	.
	.	.	.	.	.	.	.	.	1
Study IV:	.	.	.	.	.	.	.	Q0	.
	.	.	.	.	.	.	Q0	.	.

Figure 35. Major histocompatibility complex genes associating with coronary artery disease, *Chlamydia pneumoniae*, periodontitis, *Porphyromonas gingivalis*, and C3/C4 ratio. "." any allele, "–" not tested, [a] negative association (protective haplotype). See Appendix for abbreviations.

Study I: Human MHC region harbors both susceptibility (HLA-A3− B35− DR1 -related haplotypes) and protective haplotypes (HLA-B*07, HLA-DRB1*15 -related haplotype) for CAD. HLA-DR1 was alone associated with diabetes mellitus, increased LDL, smoking habit, and C4B*Q0 allele.

Study II: HLA-B*35 and -related genes were associated with the markers of *C. pneumoniae* infection. The association was even more pronounced in males and smokers carrying these genes. HLA-B*35 may be a link between chronic *C. pneumoniae* infection and CAD.

Study III: LTA+496C was associated with periodontitis in patients with CAD. HLA-DRB1*01 was associated with the elevated serum antibodies of *P. gingivalis*, suggesting that HLA-DRB1*01 associates with periodontitis in combination with *P. gingivalis*.

Study IV: The increased serum C3/C4 ratio was a novel risk marker for recurrent cardiovascular events in patients with CAD. The relative increase in serum C3 protein and decrease in C4 protein could explain changes in the C3/C4 ratio.

In conclusion, these results indicate that the CAD susceptibility haplotype (HLA-A3− B35− DR1 -related haplotypes) partially explains the development of CAD in patients possessing several recognized and novel risk factors. Among these risk factors, the telomeric part of this haplotype (e.g., HLA-B*35 allele) explains *C. pneumoniae* infection; and the centromeric part (e.g., HLA-DRB1*01 allele) explains diabetes mellitus, increased LDL, smoking, and *P. gingivalis* infection; whereas the central part of the haplotype (LTA region, e.g., LTA+496C) explains periodontitis, and the complement region (e.g., C4B*Q0) the C3/C4 ratio. Thus, all genes involved in the susceptibility haplotype have their own functional importance in CAD pathogenesis; however, HLA-DRB1*01 might be of most importance. We examined nine markers spanning the entire MHC region. Thus, it is quite possible that the observed associations in this study might be secondary to another marker(s), e.g., as has been recently shown in type I diabetes (339). Further studies are required before clear conclusions can be drawn in CAD.

13. SUMMARY

Most of the genes in the MHC region are involveed in adaptive and innate immunity, with essential function in inflammatory reactions and in protection against infections. These genes might serve as a candidate region for infection and inflammation associated diseases. CAD is an inflammatory disease. The present set of studies was performed to assess whether the MHC region harbors genetic markers for CAD, and whether these genetic markers explain the CAD risk factors: e.g., *C. pneumoniae*, periodontitis, and periodontal pathogens.

Study I was performed using two separate patient materials and age- and sex-matched healthy controls, categorizing them into two independent studies: the HTx and ACS studies. Both studies consistently showed the HLA-A3– B35– DR1 (35 ancestral haplotype) haplotype as a susceptible MHC genetic marker for CAD. HLA-DR1 alone was associated not only with CAD, but also with CAD risk factor diseases, e.g., diabetes mellitus, and hyperlipidemia. The ACS study further showed the HLA-B*07 and -DRB1*15 -related haplotype as a protective MHC haplotype for CAD.

Study II showed that patients with CAD showed signs of chronic *C. pneumoniae* infection when compared to age- and sex-matched healthy controls. HLA-B*35 or -related haplotypes associated with the *C. pneumoniae* infection markers. Among these haplotype carriers, males and smokers associated with elevated *C. pneumoniae* infection markers.

Study III showed that CAD patients with periodontitis had elevated serum markers of *P. gingivalis* and occurrence of the pathogen in saliva. LTA+496C strongly associated with periodontitis, while HLA-DRB1*01 with periodontitis and with the elevated serum antibodies of *P. gingivalis*.

Study IV showed that the increased level of C3/C4 ratio was a new risk factor and was associated with recurrent cardiovascular end-points. The increased C3 and decreased C4 concentrations in serum explained the increased level of the C3/C4 ratio. Both the higher than cut-off value (4.53) and the highest quartile of the C3/C4 ratio were also associated with worst survival, increased end-points, and C4 null alleles. The presence of C4 null alleles associated with decreased serum C4 concentration, and increased C3/C4 ratio.

In conclusion, the present studies show that the CAD susceptibility haplotype (HLA-A3− B35− DR1 -related haplotypes, Study I) partially explains the development of CAD in patients possessing several recognized and novel risk factors: diabetes mellitus, increased LDL, smoking, C4B*Q0, *C. pneumnoiae*, periodontitis, *P. gingivalis*, and complement C3/C4 ratio (Study II, III, and IV).

14. FUTURE PERSPECTIVES

The results obtained from this study will be replicated in a large independent patient cohort. If the results are replicable, it may provide an exciting possibility to develop a new diagnostic test including the MHC haplotype combination with the complement C3/C4 ratio to recognize the high risk patients at early stage, and guidelines for the development of a vaccine against *C. pneumoniae* and *P. gingivalis* infection.

15. TIIVISTELMÄ (FINNISH SUMMARY)

Human leukocyte antigen (HLA)-geenit sijaitsevat kromosomin 6 Major Histocompatibility Complex (MHC)-alueella. Monet näistä geeneistä säätelevät hankittua ja luonnollista immuunivastetta sekä tulehdusreaktioita. HLA-geenit sopivatkin ehdokasgeeneiksi monille tulehdus- ja infektiosairauksille, myös sepelvaltimotaudille. Tämän väitöskirjan tavoitteena oli selvittää altistavatko MHC-alueen geenit tai geenipoikkevuudet sepelvaltimotaudille. Lisäksi haluttiin tutkia onko sepelvaltimotautiin liittyvillä tulehduksilla (*Chlamydia pneumoniae* -bakteeri tai hampaan juurikalvon tulehduksella) ja MHC-alueen geeneillä yhdessä yhteyttä sepelvaltimotautiin.

Kirjan ensimmäisessä osatyössä käytettiin kahta potilasmateriaalia, jossa ensimmäisessä tutkittiin eri indikaatioilla hoidettuja sydänsiirtopotilaita ja toisessa vertailtiin akuutti koronaarisyndroomapotilaita ikä- ja sukupuolivakioituihin kontrolleihin. Molemmat tutkimukset osoittivat johdonmukaisesti, että haplotyyppi HLA-A3–B35–DR1 altistaa sepelvaltimotaudille. Tämän lisäksi osoitettiin, että HLA-DR1 liittyi sepelvaltimotaudin lisäksi myös sen riskitekijöihin, kuten diabetekseen, veren korkeaan kolesterolipitoisuuteen ja tupakointiin sekä C4B-puutokseen. Akuutti koronaarisyndrooma -työssä todettiin myös, että sepelvaltimotaudilta suojaavissa haplotyypeissä olivat HLA-B*07 ja -DRB1*15 alleelit.

Toisessa osajulkaisussa tutkittiin *Chlamydia pneumoniae*-infektion yhteyttä HLA-alueeseen ja sepelvaltimotautiin. *Chlamydia pneumoniae* vasta-ainemääriä vertailtiin akuutti koronaarisyndrooma-potilaiden ja ikä- ja sukupuolivakioitujen verrokkien välillä. Tutkimus osoitti, että HLA-B*35 liittyi sepelvaltimotautipotilailla *Chlamydia pneumoniae*-infektioon. Lisäksi todettiin, että miessukupuoli ja tupakointi voimistivat tätä yhteyttä. HLA-B*35 saattaa olla yhteinen tekijä sepelvaltimotaudin ja *Chlamydia.pneumoniae*-infektion välillä.

Kolmas osatyö keskittyi sepelvaltimotautipotilaisiin, joilla oli hampaan juurikalvon tulehdus eli parodontiitti. Aineistona oli 106 akuutti koronaarisyndroomapotilasta, joiden hampaat oli tutkittu sairaalaan tulovaiheessa panoramisella tomografialla ja potilaista oli otettu sylki- ja seeruminäytteet. Syljestä viljeltiin bakteerit, seerumista määritettiin muun muassa *Porphyromonas gingivalis* vasta-aineet ja parodontiiti määritettiin tomografialla. Löydöksiä verrattiin MHC-alueen geeneihin. Tärkeimpänä löydöksenä oli se, että LTA+496C tekijä assosioitui parodontiittiin. HLA-DRB1*01 esiintyi puolestaan potilailla, joilla oli sekä paradontiittia että *Porphyromonas gingivalis* -vasta-ainetta seerumissa.

Neljännessä osatyössä selvitettiin akuutti koronaarisyndroomapotilailla seerumin komplementtiproteiinien C3 ja C4-suhteen (C3/C4-suhde) merkitystä sepelvaltimotautikohtausten uusiutumiseen. Tutkimuksessa pystyttiin osoittamaan, että kohonnut C3/C4-suhde on uusi riskitekijä uusivalle akuutille

koronaarisyndroomalle. Sekä kohonnut C3- että alentunut C4 -pitoisuuden vaikuttivat kohonneeseen C3/C4-suhteeseen. Tämän lisäksi C4-puutokset alentuneen C4 -pitoisuus kanssa nostivat C3/C4-suhdetta.

Väitöskirjan työt osoittivat, että haplotyyppi HLA-A3– B35– DR1 altistaa sepelvaltimotaudille ja liittyy niin perinteisiin kuin uusiin sepelvaltimotaudin riskitekijöihin, joita ovat mm. diabetes, veren suuri kolesterolipitoisuus, tupakointi, C4B*Q0-alleeli, *Chlamydia pneumoniae*, paradontiitti, *Porphyromonas gingivalis*, sekä komplementin C3/C4 -suhde.

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Helsinki, April 2008
Anil Palikhe, MD

17. APPENDIX

Footnotes for tables:

Table 3. Recently identified susceptibility genes/loci for coronary artery disease in genome wide scan studies. ALOX5AP = Arachidonate 5-lipoxygenase-activating protein, FDFT1 = Farnesyl-diphosphate farnesyltransferase 1, FGL1 = Fibrinogen-like 1, GATA1 = GATA binding protein 1, LTA = Lymphotoxin alpha, MEF2A = Monocyte enhancer factor 2A, MI = Myocardial infarction, MSR1 = Macrophage scavenger receptor 1, MTHFD1L = Methylenetetrahydorfolate dehydrogenase (NADP+ dependent) 1-like, NADP = Nicotinamide andenine dinucleotide phosphate, PDE4D = Phosphodiesterase 4D, WTCCC = Wellcome Trust case-control consortium.

Table 4. MHC genes and polymorphisms in MHC regulating loci/or associating genes linking to coronary artery disease. C4 = Complement component C4, HFE = Hemochromatosis, HLA = Human leukocyte antigens, HSP70-1 = Heat shock 70 kDa protein 1, LGALS2 = Lectin galactoside-binding soluble 2, LTA = Lymphotoxin alpha, MHC2TA = Class II MHC transactivator (also known as CIITA), TNF-α = Tumor necrosis alpha.

Table 5. Gene clusters within the extended major histocomapatibility compelx region. Bf = Complement factor B, C2 = Complement component C2, C4 = Complement component C4, HLA = Human leukocyte antigen, LTA = Lymphotoxin alpha, MICA = Major histocompatiblity complex class I polypeptide-related sequence A, MICB = Major histocompatiblity complex class I polypeptide-related sequence B, TNF-α = Tumor necrosis factor alpha, tRNA = transfer ribonucleide acid.

Table 6. Categories of immune response genes in the extended major histocompatibility complex region. AIF1 = Allograft inflammatory factor 1, BF = Complement factor B, BTN1A1 = Butyrophilin subfamily 1 member A1, BTN2A1 = Butyrophilin subfamily 2 member A1, BTN2A2 = Butyrophilin subfamily 2 member A2, BTN2A3 = Butyrophilin subfamily 2 member A3, BTN3A1 = Butyrophilin subfamily 3 member A1, BTN3A2 = Butyrophilin subfamily 3 member A2, BTN3A3 = Butyrophilin subfamily member A3, BTNL2 = Butyrophilin-like 2, C2 = Complement component C2, C4A = Complement component C4A, C4B = Complement component C4B, C6orf25 = Chromosome 6 open reading frame 25, DAXX = Death-associated protein 6, DDAH2 = Dimethylargininen dimethylaminohyddrolase 2, FKBPL = FK506 binding protein-like, HFE = Hemochromatosis, HLA = Human leukocyte antigen, HSPA1A = Heat shock 70 kDa protein 1A, HSPA1B = Heat shock 70 kDa protein 1B, HSPA1L = Heat shock 70 kDa protein 1-like, IER3 = Immediate early response 3, LST1 = Leukocyte specific transcript 1, LTA = Lymphotoxin alpha, LTB = Lymphotosin beta, LY6G5B = Lymphocyte antigen 6 complex locus G5B, LY6G5C = Lymphocyte antigen 6 complex locus G5C, LY6G6C = Lymphocyte antigen 6 locus G6C, LY6G6D = Lymphocyte antigen 6 complex locus G6D, LY6G6E = Lymphocyte antigen 6 complex locus G6E, MICA = Major histocompatiblity complex class I polypeptide-related sequence A, MICB = Major histocompatibility complex class I polypeptide-related sequence B, MOG = Myelin oligodendrocyte glycoprotein, NCR3 = natural cytotoxicity triggering receptor 3, NFKBIL1 = Nuclear factor kappa light polypeptide gene enhance in B-cell, PRSS16 = Protease serin 16, PSMB8 = Proteosome subunit beta type 8, PSMB9 = Proteosome subunit beta type 9, RXRB = Retinoid X receptor beta, TAP1 = Transpoerter 1, TAP2 = Transporter 2, TAPBP = TAP binding protein, TNF = Tumor necrosis factor, UBD = Ubiquitin D.

Table 8. Laboratory methods used in the present study. *A. actinomycetemcomitans* = *Aggregatibacter actinomycetemcomitans* (previsously known as *Actinobacillus actinomycetemcomitans*), ACS = Acute coronary syndrome, *C. pneumoniae* = *Chlamydia pneumoniae*, C4A = Complement component C4A, C4B = Complement component C4B, DNA = Deoxyribonucleic acid, ELISA = Enzyme-linked immunosorbent assay, HLA = Human leukocyte antigen, IgA = Immunoglobulin A, IgG = Immunoglobulin G, LTA = Lymphotoxin alpha, *P. gingivalis* = *Porphyromonas gingivalis*, PCR = Polymerase chain reaction, SNP = Single nucleotide polymorphism, SSP = Sequence specific primer.

Footnotes for figures:

Figure 4. Migration of monocytes and T cells into intima, and formation of foam cells and activation of macrophages. HSP60 = Heat shock 60 kDa protein, LDL = Low density lipoprotein, LPS = Lipopolysaccharide, M-CSF = Colony stimulating factor 1 (macrophage), oxLDL = Oxidized LDL, TLR = Toll-like receptor, VCAM1 = Vascular cell adhesion molecule 1, VLA4 = Very large antigen 4.

Figure 6. Toll-like receptor type 4 signaling pathway and its relation with atherosclerosis. EDA = Extra domain A in fibronectin, HSP60 = Heat shock 60 kDa protein, ICAM = Intercellular adhesion molecule, IFN = Interferon, IL =

Interleukin, IP-10 = IFN-γ inducible protein 10, IRF3 = Interferon regulatory factor 3, LPS = Lipopolysaccharide, MCP-1 = Monocyte chemoattractant protein 1, MM-LDL = Minimally-modified low density lipoprotein, MMP = Matrix metalloproteinase, NF-κB = Nuclear factor kappa B, RANTES = Regulated upon activation, normal T-cell expressed and secreted, VCAM = Vascular cell adhesion molecule.

Figure 7. T cell responses in atherosclerosis (A), activation of Th1 cell (B), and Th2 (B), and Treg cell (C). CD4 = CD4 molecule, CD40 = CD40 molecule, CD40L = CD40 ligand, HSP60 = Heat shock 60 kDa protein, IFN-γ = Interferon gamma, IL = Interleukin, MHC = Major histocompatibility complex, oxLDL = Oxidized low density lipoprotein, TCR = T-cell receptor, TGF-β = Transforming growth factor beta, Th = T helper cell, TNF = Tumor necrosis factor, Treg = T regulatory cell.

Figure 11. Effects of *Chlamydia pneumoniae* on atheroma. CPn = *Chlamydia pneumoniae*, HSP60 = Heat shock 60 kDa protein, ICAM-1 = Intercellular adhesion molecule 1, IL = Interleukin, LDL = Low density lipoprotein, LPS = Lipoprotein, oxLDL = Oxidized low density lipoprotein, VCAM-1 = Vascular cell adhesion molecule 1.

Figure 17. Tumor necrosis factor alpha and lymphotoxin alpha receptor specificity. LTA = Lymphotoxin alpha, LTα1β2 = 1 unit of lymphotoxin alpha and 2 units of lymphotoxin beta, LTα2β1 = 2 units of lymphotoxin alpha and 1 unit of lymphotoxin beta, LTβR = Lymphotoxin beta receptor, p55 = Protein P55, p75 = Protein P75, TNF-α = Tumor necrosis factor alpha, TNFR1 = Tumor necrosis factor receptor superfamily member 1A, TNFR2 = Tumor necrosis factor receptor superfamily member 1B.

Figure 18. Model of complement cascade. C1q = q subunit of complement component C1, C1r = r subunit of complement component C1, C1s = s subunit of complement component C1, C2 = Complement component C2, C3 = Complement component C3, C3a = a subunit of complement component C3, C3d = d subunit of complement component C3, C4 = Complement component C4, C4a = a subunit of complement component C4, C4b = b subunit of complement component C4, C5 = Complement component C5, C5a = a subunit of complement component C5, C6 = Complement component C6, C7 = Complement component C7, C8 = Complement component C8, C9 = Complement component C9, iC3b = Inactivated C3b, MAC = Membrane-attack complex, MASP1 = Mannan-binding lectin serin peptidase 1, MASP2 = Mannan-binding lectin serin peptidase 2, MBL = Mannan-binding lectin.

Figure 20. Location and copy number variations of the RP-C4-CYP21-TNX. 1C7 = also known as natural cytotoxicity triggering receptor 3 NCR3, AIF1 = Allograft inflammatory factor 1, B144 = also known as leukocyte specific transcript 1 LST1, BAT1 = HLA-B associated transcript 1, Bf = Complement factor B, C2 = Complement component C2, C4A = Complement component C4A, C4B = Complement component C4B, CYP21A = Cytochrome P450 21 A psuedogene (also known as CYP21A1P), CYP21B = Cytochrome P450 21-hydroxylase (also known as CYP21A2), HSP70 = Heat shock 70 kDa protein, IkBL = Inhibitor of kappa B-like, LTA = Lymphotoxin alpha, LTB = Lymphotoxin beta, NOTCH4 = NOTCH homolog 4 (drosophila), PBX2 = Pre-B-cell leukemia homeobox 2, PIPP9 = Peptidylprolyl isomerise A pseudogene 9, RAGE = Advanced glycosylation end product-specific receptor (also known as: AGER, advanced glycosylation end product-specific receptor), RP1 = also known as serin/threonine kinase 19 STK19, RP2 = also known as serin/threonine kinase 19 pseudogene STK19P, SKIV2L = Superkiller viralicidic activity 2-like, TNF = Tumor necrosis factor, TNXA = Tenascin XA pseudogene, TNXB = Tenascin XB.

Figure 21. A schematic diagram of genes in the major histocompatibility complex region. 1C7 = also known as natural cytotoxicity triggering receptor 3 NCR3, AIF1 = Allograft inflammatory factor 1, B144 = also known as leukocyte specific transcript 1 LST1, BAT1 = HLA-B associated transcript 1, Bf = Complement factor B, BTN = Butyrophilin genes, BTNL2 = Bytyrophilin like 2, C2 = Complement component C2, C4A = Complement component C4A, C4B = Complement component C4B, C6orf10 = Chromosome 6 open reading frame 10, C6orf40 = Chromosme 6 open reading frame 40, COL11A2 = Collagen type XI alpha 2, CYP21A = Cytochrome P450 21 A psuedogene (also known as CYP21A1P), CYP21B = Cytochrome P450 21-hydroxylase (also known as CYP21A2), DAXX = Death-associated protein 6, HFE = Hemochromatosis, HCG24 = Human leukocyte antigen complex group 24, HIST = Histone cluster genes, HIST1H2AA = Histone cluster 1 H2aa, HLA = Human leukocyte antigens, HSP70 = Heat shock 70 kDa protein, IkBL = Inhibitor of kappa B-like, LTA = Lymphotoxin alpha, LTB = Lymphotoxin beta, MICA = Major histocompatibility complex class I polypeptide-related sequence A, MICB = Major histocompatiblity complex class I polypeptide-related sequence B, MOG = Myelin oligodendrocyte glycoprotein, NOTCH4 = NOTCH homolog 4 (drosophila), OR = Olfactory receptor genes, PBX2 = Pre-B-cell leukemia homeobox 2, PIPP9 = Peptidylprolyl isomerise A pseudogene 9, RAGE = Advanced glycosylation end product-specific receptor (also known as: AGER, advanced glycosylation end product-specific receptor), RING1 = Ring finger protein 1, RP1 = also known as serin/threonine kinase 19 STK19, RP2 = also known as serin/threonine kinase 19 pseudogene STK19P, RPL12P1 = Ribosomal protein L12 psudogene 21, SKIV2L = Superkiller viralicidic activity 2-like, TAP = Transporter genes,

TAPBP = TAP binding protein, TNF = Tumor necrosis factor, TNXA = Tenascin XA pseudogene, TNXB = Tenascin XB.

Figure 23. Major histocompatibility complex class I molecule – peptide – T cell receptor complex (A), and independent contribution of each amino acid of peptides in binding (B). β2m = β2-microglobulin, CDR1 = T cell receptor complementary-determining region 1, CDR2 = T cell receptor complementary-determining region 2, CDR3 = T cell receptor complementary-determining region, MHC = Major histocompatibility complex.

Figure 25. Immune responses by major histocompatibility complex molecules. CD8 = CD8 molecule, IFN-γ = Interferon gamma, IL = Interleukin, MHC = Major histocompatiblity complex, NK cell = Natural killer cell, TGF-β = Transforming growh factor beta, Th1 = T helper cell type 1, Th2 = T helper cell type 2, Treg = T regulatory cell.

Figure 26. Activation or silencing of class II major histocompatibility complex transactivator gene. BCG = Bacille Calmette-Guérin, DM = Human leukocyte antigen DM locus, DO = Human leukocyte antigen DO locus, HIV = Human immunodeficiency virus, IFN-γ = Interferon gamma, Ii = Major histocompatibility complex class II invariant chain, IL = Interleukin, MHC = Major histicompatibility complex, MHC2TA = Class II major histocompatibility complex transactivator, MTB = Mycobacterium tuberculosis, NF-Y = Nuclear factor Y, NO = Nitric oxide, RFX = Regulatory factor X, SOCS-1 = Suppressor of cytokine signaling-1, TGF-β = Transforming growth factor beta, X2BP = X2-binding protein.

Figure 28. Approximate localization of studied genetic markers. C4A = Complement component C4A, C4B = Complement component C4B, HLA = Human leukocyte antigen, LTA = Lymphotoxin alpha, MHC = Major histocompatibility complex.

Figure 35. Major histocompatibility complex genes associating with coronary artery disease, *Chlamydia pneumoniae*, periodontitis, *porphyromonas gingivalis*, and C3/C4 ratio. C4A = Complement componet C4A, C4B = Complement component C4B, CAD = Coronary artery disease, *C. pneumoniae* = *Chlamydia pneumoniae*, HLA = Human leukocyte antigen, HTx = Heart transplant, LTA = Lymphotoxin alpha, *P. gingivalis* = *Porphyromonas gingivalis*, Q0 = Quantitative null allele.

18. TABLES AND FIGURE LEGENDS

Tables:

Figures:

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